**DAMIANA DINIZ ROSA**

# EVALUATION OF KEFIR CONSUMPTION ON METABOLIC, IMMUNE, HORMONAL AND HISTOLOGICAL PARAMETERS IN SPONTANEOUSLY HIPERTENSIVE RATS WITH INDUCED METABOLIC SYNDROME

Thesis presented to the Federal University of Viçosa, as partial fulfillment of the requirements from the Graduate Program in Nutrition Sciences for attaining the degree of *Doctor Scientiae*.

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# EVALUATION OF KEFIR CONSUMPTION ON METABOLIC, IMMUNE, HORMONAL AND HISTOLOGICAL PARAMETERS IN SPONTANEOUSLY HYPERTENSIVE RATS WITH INDUCED METABOLIC SYNDROME

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"... a mente que se abre a uma nova idéia...

... jamais voltará ao seu tamanho original..."

*Albert Einstein*

*"Renda-se como eu me rendi.*
*Mergulhe no que você não conhece como eu mergulhei.*
*Não se preocupe em entender, viver ultrapassa qualquer entendimento."*

*(Clarice Lispector)*

*Dedico esta tese aos meus pais Jairo e Helena, aos meus irmãos e ao meu amor, Keller com carinho e gratidão.*

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**SUMÁRIO**

## RESUMO

ROSA, Damiana Diniz, D.Sc., Universidade Federal de Viçosa, Março, 2014. **Avaliação do consumo de Kefir nos parâmetros metabólicos, imunes, hormonais e histológicos de ratos espontaneamente hipertensos induzidos à síndrome metabólica**. Orientador: Maria do Carmo Gouveia Peluzio. Coorientadores: Célia Lúcia de Luces Fortes Ferreira e Alessandra Barbosa Ferreira Machado.

O interesse por bebidas lácteas enriquecidas com probióticos tem aumentado nos últimos anos, decorrente da crescente demanda por alimentos seguros e com potencial de reduzir o risco de doenças crônicas. O kefir é uma bebida fermentada produzida a partir de uma cultura inicial produzida principalmente a partir do leite. Esta é amplamente conhecida e traz consigo uma longa história de propriedades benéficas à saúde. O kefir pronto para o consumo apresenta aspecto viscoso, ácido e levemente alcoólico, com uma mistura única de microorganismos que exerce ação probiótica com efeitos benéficos na prevenção de doenças. O objetivo do presente estudo foi avaliar o efeito da suplementação de kefir em ratos espontaneamente hipertensos (SHR) induzidos à síndrome metabólica (MetS) com glutamato monossódico (MSG) e avaliar o efeito protetor do kefir sobre os parâmetros metabólicos, hormonais, imunes e histológicos. Para a indução da MetS, 20 ratos com 2 dias de vida receberam cinco injeções intradérmicas de MSG (MSG, 4 mg/g peso corporal). Para o controle negativo, 10 animais receberam cinco injeções intradérmicas de solução salina (0,9% NaCl). Os animais foram mantidos em gaiolas coletivas por 100 dias com água e dieta add libitum para o desenvolvimento da MetS. Após este período, os animais com MetS foram divididos em dois grupos experimentais: PC, controle positivo (1mL 0,9% NaCl/ dia) e grupo kefir (1mL kefir/dia). O grupo NC, composto pelos 10 animais sem indução da MetS, receberam 1mL 0,9% NaCl/ dia, durante o período experimental. Os tratamentos foi realizado via gavagem. Os animais foram alojados em gaiolas individuais e mantidos em condições padronizadas por 10 semanas. Foram avaliados os seguintes parâmetros: peso corporal, ingestão alimentar, marcadores da obesidade, pressão arterial, parâmetros metabólicos, teste de tolerância oral à glicose, resistência à insulina e níveis plasmáticos de lipopolissacarídeos. No tecido adiposo intra-abdominal, foram avaliadas citocinas inflamatórias e as análises histológicas foram realizadas para investigar a hipertrofia e hiperplasia dos adipócitos. No fígado, foram realizadas análises do teor lipídico, quantificação de enzimas antioxidantes e produtos de oxidação. No intestino delgado e colon, as análises histológicas foram realizadas para avaliar a integridade da mucosa intestinal. Nas fezes, foram extraídos os ácidos graxos de cadeia curta e mensurado o

pH fecal. A permeabilidade intestinal foi avaliada por meio do teste lactulose-manitol antes e após as 10 semanas de consumo do kefir. Adicionalmente, um outro ensaio experimental foi realizado para avaliar a toxicidade subcrônica do kefir, utilizando ratos Wistar. Os animais foram distribuídos em três grupos experimentais (n = 6 grupo) e receberam via gavagem: Grupo Controle (0,7 mL de água); Grupo Kefir (0,7 mL de kefir /dia, normodose) e Grupo HKefir (grupo sobredose que recebeu 3,5ml/dia de kefir, dose 5 vezes maior). Os animais foram mantidos em gaiolas individuais e em condições padronizadas durante 4 semanas. Os animais foram avaliados quanto ao crescimento, parâmetros metabólicos, potencial de infectividade e patogenicidade. Os animais induzidos a MetS e suplementados com kefir apresentaram redução dos níveis de triglicerídeos plasmáticos, lipídios e triglicerídeos hepáticos, redução da resistência à insulina, glicemia e insulina de jejum, além da redução nos marcadores da obesidade (circunferência torácica e abdominal). No tecido adiposo, houve redução da citocina pró-inflamatória IL1-β e aumento da citocina anti-inflamatória IL-10. A ação antioxidante do kefir foi verificada no tecido hepático dos animais, devido aos menores níveis de malondialdeído (MDA) e hidroperóxidos. Menor permeabilidade intestinal e níveis plasmáticos de lipopolissacarídeos foram verificados nos animais que receberam kefir. Maiores níveis de ácidos graxos de cadeia curta foram encontrados nas fezes dos animais suplementados com kefir. A administração neonatal de MSG foi capaz de aumentar a área dos adipócitos no tecido adiposo e reduzir a área das ilhotas, no tecido pancreático. O consumo de Kefir não foi capaz de reduzir a pressão arterial dos animais. O ensaio de toxicidade mostrou que a normodose e alta dose do kefir não influenciou no crescimento, parâmetros metabólicos, potencial de patogenicidade e infectividade, demonstrando que o consumo de kefir é seguro. Diante dos resultados encontrados, o kefir é um alimento potencial no controle da MetS, pois foi capaz de reduzir importantes parâmetros determinantes para a ocorrência de síndrome metabólica: resistência a insulina, triglicerídeos plasmáticos, adiposidade central, estresse oxidativo e marcadores inflamatórios. Estes achados indicam que o kefir é um alimento seguro e constitui um alimento potencial no controle/ prevenção da MetS.

## ABSTRACT

ROSA, Damiana Diniz, D.Sc., Universidade Federal de Viçosa, March 2014. **Evaluation of kefir consumption on metabolic, immune, hormonal and histological parameters in spontaneously hipertensive rats with induced metabolic syndrome.** Adviser: Maria do Carmo Gouveia Peluzio. Co-Advisers: Célia Lúcia de Luces Fortes Ferreira and Alessandra Barbosa Ferreira Machado.

The interest in fermented milk beverages containing probiotics has greatly increased over the last few years because of the increasing demand for safe and high quality products. The consumption of kefir has now spread throughout the world, spurred by its long history of beneficial health effects. Kefir is a fermented dairy product, manufactured by starter culture. The final product is a viscous, acidic, and mildly alcoholic milk drink which contains a unique mixture of microorganisms that can be beneficial in the maintenance of many disorders and diseases. The aim of the present study was to assess the effect of kefir supplementation in spontaneously hypertensive rats (SHR) with induced metabolic syndrome (MetS) and to evaluate the protective effect of kefir on metabolic, immune, hormonal and histological parameters. For the induction of MetS, 20 two-day-old male rats received intradermal injections of monosodium glutamate (MSG, 4 mg/g body weight, Sigma Co., St. Louis, MO), until they completed the age of six days, which gave a total of five applications. For a negative control (NC), 10 two-day-old male rats received intradermal injections of saline solutions (0.9 % NaCl/day) in the same conditions. After weaning, all the animals were housed in collective cages for 100 days with food and water add libitum until the MetS was developed. The rats with induced MetS were randomly divided in two groups (10 animals in each group): positive control (PC, 1mL 0.9 % sodium chloride solution/day) and kefir group (1mL kefir/day). Ten animals without induced MetS served as negative control (NC, 1mL 0.9 % sodium chloride solution/day). Feeding was carried out by gavage. The animals were housed in individual cages and maintained under standard conditions. Study lasted 10 weeks. The following parameters were evaluated: body weight, food intake, obesity markers, arterial blood pressure, biochemical analyses, oral glucose tolerance tests, insulin resistance, and levels of plasma lipopolysaccharide. In intra-abdominal adipose tissue, inflammatory cytokines and histological analyses were performed to evaluate hypertrophy and hyperplasia of adipocytes. In liver, levels lipids, antioxidant enzymes and oxidation products in hepatic tissue were measured. In small intestine and colon, histologic analyses were performed to assess the integrity of the intestinal mucosa. In feces, the short chain fatty acids were

extracted and the fecal pH was measured. Intestinal permeability was assessed by lactulose-mannitol test before and after 10 weeks of kefir consumption. Moreover, another parallel experimental trial was conducted to evaluate the subchronic toxicity of kefir in Wistar rats. The animals were divided into three experimental groups (n = 6 group): Wistar rats were randomly divided into three groups (n=6/group): control group received 0.7 mL of water, kefir group received 0.7 mL/day of kefir, (normodose), and Hkefir group received 3.5 mL/day of kefir (fivefold higher dose). Feeding was carried out by gavage. The animals were housed in individual cages and maintained under standard conditions for 4 weeks. The animals were evaluated for growth, metabolic parameters, potential infectivity and pathogenicity. nimals with MetS and supplemented with kefir presented reduced levels of plasma triglycerides, hepatic lipids, hepatic triglycerides, reduced insulin resistance, decreased levels of blood glucose, fasting insulin and markers of obesity (abdominal and thoracic circumference). Adipose tissue demonstrated reduction of proinflammatory cytokine IL1-β and increased anti-inflammatory cytokine IL-10. The antioxidant activity of kefir was observed in the liver tissue of animals due to lower levels of malondialdehyde (MDA) and hydroperoxides. Decreased intestinal permeability and plasma lipopolysaccharide levels were observed in animals supplemented with kefir. In addition, higher levels of short-chain fatty acids were found in the feces of animals supplemented with kefir. The neonatal administration of MSG was able to increase the area of adipocytes in adipose tissue and reduce the area of the islets in the pancreatic tissue. Kefir consumption was unable to reduce the blood pressure of the animals. The toxicity test showed that the high dose and normal dose of kefir had no influence on growth, metabolic parameters, the potential pathogenicity and infectivity, demonstrating that kefir consumption is safe. Taken together, kefir can be considered a potential tool to control MetS because it was able to reduce important determinants for the occurrence of metabolic syndrome, i.e. insulin resistance, plasma triglycerides, central adiposity, oxidative stress and inflammatory markers. These findings indicate that kefir is a safe probiotic food and has a potential in the control and prevention of MetS.

# 1. GENERAL INTRODUCTION

Kefir is a fermented dairy product, manufactured by starter culture. Nowadays the kefir beverage is consumed widely around the world. Kefir has its origin in the Caucasus, Tibetan, or Mongolian mountains, where at more than 2000 years BC the grains were already traditionally passed down from generation to generation among the Caucasus tribes, being considered a source of family wealth. The name kefir originates from the Slavic *Keif*, meaning "well-being" or "living well", due to the overall sense of health and well-being generated in those who consume it [1]. Kefir differs from other fermented products in that it is produced from kefir grains that comprise a specific and complex mixture of lactic acid- and acetic acid-producing bacteria, and lactose-fermenting and non-fermenting yeast, which live in a symbiotic association [2]. Kefir grains, when inoculated into a culture medium such as milk, produce an acidified fermented beverage that is slightly carbonated and contains small amounts of alcohol. During the fermentation, lactic acid, bioactive peptides, exopolysaccharides, antibiotics, and numerous bacteriocins are produced [1, 3].

According to the *Codex Alimentarius* (Codex Stan 243-2003), a typical kefir should contain at least 2.7% protein, 0.6% lactic acid, and less than 10% fat. The percentage of alcohol is not established. The total number of microorganisms in the beverage should be at least $10^7$ CFU/mL and the yeast number not less than $10^4$ CFU/mL [4].

Kefir has raised interest in the scientific community due to its health benefits in fighting numerous infections and diseases [5]. The product is considered a probiotic food because it contains live microorganisms that confer health benefits when administered in appropriate numbers [6]. Moreover, the microbial strains characterized in milk kefir often belong to species (lactobacilli, bifidobacteria, *Saccharomyces)* whose health benefits have been well characterized [7-10]

The beneficial properties of kefir include antimicrobial [11, 12]; anti-inflammatory [13-15]; anti-allergenic [13]; immune system modulation [16, 17]; healing activity [18]; hypocholesterolemic [19, 20]; antioxidant [21, 22]; control of lactose intolerance [23]; beneficial effect on gut health [23, 24]; hypotensive [25];

anticarcinogenic [26, 27], reduced progression of renal injury [22], improved fatty liver syndrome [28] and plasma glucose control [29].

The beneficial effects of kefir in the control of metabolic parameters that are directly related to obesity and the metabolic syndrome (MetS), such as lowering cholesterol and plasma triglycerides, control of glucose metabolism and insulin resistance, reduced pressure blood, anti-inflammatory and antioxidant action. However, there are no studies that relate consumption of kefir in the treatment / prevention of obesity and MetS.

The MetS is a constellation of overweight/obesity, hypertension, and disturbances of lipid and carbohydrate metabolism. Each component of MetS is a known risk factor for the development of type 2 diabetes, atherosclerosis, and coronary artery disease [30]. It has been reported that the association of MetS with cardiovascular disease increases total mortality 1.5 times and cardiovascular death 2.5 times. People with MetS also have a 5-fold higher risk of developing type 2 diabetes [31]. It has been demonstrated that the prevalence of MetS is increasing worldwide, and for the adult population is estimated to be about 20 to 25%, largely the result of greater obesity and sedentary lifestyles [32].

The epidemic of obesity is now recognized as one of the most important public health problems facing the world today. In this context, it is important to find alternatives to traditional therapies for the treatment of obesity and MetS. Functional foods and probiotics appear as a dietotherapeutic alternative to the management of MetS. Probiotics promote benefits that go beyond merely nourish, but also modulate the intestinal microbiota and reduce subchronic inflammation. There are many mechanisms to be clarified and studies are needed to elucidate the effects of probiotics / prebiotics and even antibiotics as adjuncts in the treatment of obesity, diabetes mellitus and MetS.

Milk kefir is a functional food with a great potential. There are few researches directed to the kefir in the context of MetS. Numerous observed physiological effects support the health-promoting benefits of kefir. However, different study designs of clinical trials on kefir cause difficulties in drawing clear conclusions. In addition, it has been recently found that different manufacturing conditions alter the original characteristics of probiotics [33], which therefore may influence their physiological effect on health.

The choice of kefir as an object of study is justified by being a low cost probiotic, easily produced indoors and incorporated into the diet. Therefore, the kefir

hasa great potential for use in clinical practice in the prevention and treatment of chronic diseases.Thus, the objective of this study is to evaluate the effects of subchronic consumption of kefir in metabolic syndrome - induced SHR rats on metabolic, hormonal and immune parameters. Additionally, we conducted a toxicity study in rats to assess safe levels of kefir consumption.

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# 3. OBJECTIVES

## 3.1. General Objective

Evaluation of kefir consumption on metabolic, immune, hormonal and histological parameters in SHR rats with induced metabolic syndrome.

## 3.2. Specific Objectives

1) To review published research on the kefir probiotic, with the focus on its nutritional and microbiological compositions, and beneficial effects on human and animal health.

2) To evaluate the effect of kefir supplementation in MSG-induced MetS in SHR animals and determine the protective effect of kefir on physiological and histological parameters, inflammatory markers, oxidative stress and glycemic index control in animal model.

3) To determine the protective effect of kefir on animal model of MSG-induced metabolic syndrome and on the control of metabolic parameters, intestinal permeability, plasmatic lipopolysaccharide determination, short-chain fatty acids in feces, and histological and morphometric analysis in the bowel.

4) To conduct a subchronic toxicity study using Wistar rats, offering to the animals different doses of kefir to evaluate: growth, hematology and blood chemistry, as well as the potential infectivity and pathogenicity (translocation and mucosal histology) of kefir.

## 4. RESULTS:

- Article 1 Review: “Health benefits of milk kefir”

- Article 2 Original Article: “Kefir reduces insulin resistance and inflammatory cytokine expression in animal model of the metabolic syndrome “

- Article 3 Original Article: “Kefir reduces endotoxemia, intestinal permeability and increase fecal short-chain fatty acids of rats with induced metabolic syndrome”

- Article 4 Original Article: "Subchronic toxicity study of Kefir by oral administration in Wistar rats"

# 5. ARTICLE 1: Health benefits of milk kefir

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**Running title:** Beneficial effects of kefir

**Abbreviations:** Metabolic Syndrome (MetS); Lactic Acid Bacteria (LAB); Short Chain Fatty Acids (SCFA); Angiotensin Converting Enzyme (ACE), gastrointestinal tract (GIT); hydroxymethylglutaroyl coenzyme A (HMG-CoA).

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## 5.1. Abstract

Kefir drink is produced from kefir grains that comprise a specific and complex mixture of microorganisms that live in a symbiotic association. The long history of kefir consumption is common in some European and Asian countries. However, it is only recently that kefir has raised interest in the scientific community due to its wide spectrum of activity and numerous positive effects on health. Regular drinking of kefir is associated with health benefits, including anticarcinogenic, hypocholesterolemic, immunomodulatory, antioxidant, antihypertensive, anti-inflammatory, and antimicrobial effects and healing, among others. Published research studies provide new clues to the use of this functional food in health maintenance. They also indicate that further work is needed to investigate the beneficial effect of kefir and to reveal its complex mechanisms of action. This review focuses on the nutritional and microbiological composition of kefir drink and the grains. Beneficial effects of kefir in animal and human health and disease are also extensively reviewed.

**Keywords:** milk kefir, kefir grains, probiotic, functional food, fermented milk, health effect, nutrition.

**Highlights**

-Nutritional and microbiological compositions of kefir influence its physiological properties;

-Health benefits of kefir reflect its complexity;

-Kefir is a promising tool in treatment of numerous disorders;

-Kefir positively affects our well-being;

## 5.2. Introduction

Kefir has its origin in the Caucasus, Tibetan, or Mongolian mountains, where at more than 2000 years BC the grains were already traditionally passed down from generation to generation among the Caucasus tribes, being considered a source of family wealth. The name kefir originates from the Slavic Keif, meaning "well-being" or "living well", due to the overall sense of health and well-being generated in those who consume it [1]. Kefir differs from other fermented products in that it is produced from kefir grains that comprise a specific and complex mixture of lactic acid- and acetic acid-producing bacteria, and lactose-fermenting and non-fermenting yeast, which live in a symbiotic association [2]. Kefir grains, when inoculated into a culture medium such as milk, produce an acidified fermented beverage that is slightly carbonated and contains small amounts of alcohol. During the fermentation, lactic acid, bioactive peptides, exopolysaccharides, antibiotics, and numerous bacteriocins are produced [1; 3].

According to the *Codex Alimentarius* (Codex Stan 243-2003), a typical kefir should contain at least 2.7% protein, 0.6% lactic acid, and less than 10% fat. The percentage of alcohol is not established. The total number of microorganisms in the beverage should be at least $10^7$ CFU/mL and the yeast number not less than $10^4$ CFU/mL [4].

Kefir has raised interest in the scientific community due to its health benefits in fighting numerous infections and diseases [5]. The product is considered a probiotic food because it contains live microorganisms that confer health benefits when administered in appropriate numbers [6]. Moreover, the microbial strains characterized in milk kefir often belong to species (lactobacilli, bifidobacteria, *Saccharomyces)* whose health benefits have been well characterized [7; 8; 9; 10]

The beneficial properties of kefir include antibacterial [11]; anti-inflammatory [11; 12]; anti-allergenic [12]; hypocholesterolemic [13]; antioxidant [14]; control of glucose intolerance [15]; beneficial effect on gut health [15; 16]; hypotensive [17]; anticarcinogenic [18] and plasma glucose control [19]. With these in mind, we wrote the present review of published research on the kefir probiotic, with the focus on its nutritional and microbiological compositions, and beneficial effects on human and animal health.

## 5.3. Methods

Medline/PubMed, Scielo and Lilacs were searched using the following terms: kefir, kefiran, milk kefir, kefir grains, probiotic, functional food, fermented milk, health

effect, nutrition. For data searches, the terms in English, Spanish and Portuguese were used either alone or in association. Review and original articles were selected according to their titles and abstracts. Each selected manuscript was then studied critically.

### 5.4. Characteristics of kefir grains

Kefir grains have a similar shape to the cauliflower. They are elastic, irregular, gelatinous, with an ivory or white color, and variable size, from 0.3 to 3.5 cm in diameter [20; 21]**(Fig. 1)**. In general, kefir grain consists of 4.4% fat, 12.1% ash, 45.7% mucopolysaccharide, 34.3% total protein (27% insoluble, 1.6% soluble, and 5.6% free amino acids), vitamins B and K, tryptophan, calcium, phosphorus, and magnesium [22].

**Fig. 1** – Macroscopic structure of kefir grains.

The presence of D-glucose and D-galactose in a 1:1 ratio in the complex structure of polysaccharides (kefiran) is responsible for the connection between the microorganisms and kefir grains [23]. Kefiran features include viscosity, water solubility, and resistance to bowel enzymatic hydrolysis. The production of kefir is mainly related to the presence of *Lactobacillus kefiranofaciens* and *Lactobacillus kefiri* in the grains [2; 24].

In kefir grains, the peripheral portion is composed almost exclusively of bacteria, predominantly *Bacillus*, whereas the inner portion of the grain contains yeasts, and the interface of the inner and outer portions has a mixed composition, where bacteria with long polysaccharide filaments, yeasts, and fungi are found [2; 23].

### 5.5. Kefir production

Kefir beverage can be produced from whole, semi-skimmed, or skimmed pasteurized cow, goat, sheep, camel, or buffalo milk [25]. Furthermore, brown sugar aqueous solution, at a concentration from 3 to 10%, fruit juice, soy milk, and whey may be used as culture media [26]. The kefir grains or part of the drink can be added to the culture media as a starter culture [25].

Although there is an ideal relationship between the grains and the fermentation substrate (1:30 to 1:50 w/v in case of animal milk), in practice the measures are made empirically within known limits [27]. The substrate fermentation typically occurs at temperatures ranging from 8 to 25°C, in a partially closed container, at a variable time from 10 to 40 hours. The most common incubation time is, however, 24 h [16; 28; 29].

After fermentation, the grains are separated from the drink by filtration using a sieve [2]. When milk is used as a substrate, the fermented drink is similar to yogurt. The higher the fat content in the milk, the thicker and creamier the kefir [25]. Kefir grains may increase in size by up to 25% of the original to form a new biomass, which allows continuous production, since the grains can be further added to culture media [1; 20; 21]. Kefir can be consumed immediately after grain separation or can be refrigerated for later consumption. During the cooling step, alcoholic fermentation leads to the accumulation of $CO_2$, ethanol, and vitamin B complex [25; 27]. This maturation step reduces lactose content, making the product desirable for consumption by people with lactose intolerance and diabetes [27] (**Fig. 2)**.

The grains can be stored in different ways. When stored at 4°C, they are active for only 8 to 10 days. Lyophilization or drying at room temperature for 36/48 h allows maintenance of the activity for 12–18 months [20]. Wszolek and co-workers [30] proposed a conventional method of drying at a 33°C or vacuum drying to preserve the grains. However, Garrote *et al.* [31] observed that freezing at -20°C was the best method for grain preservation. Kefir grains remain stable for many years without losing their activity, if stored under favorable conditions. The process of reconstitution consists of

performing successive incubations in milk. The grains slowly reestablish their structure and subsequently, new kefir grains are formed [32].

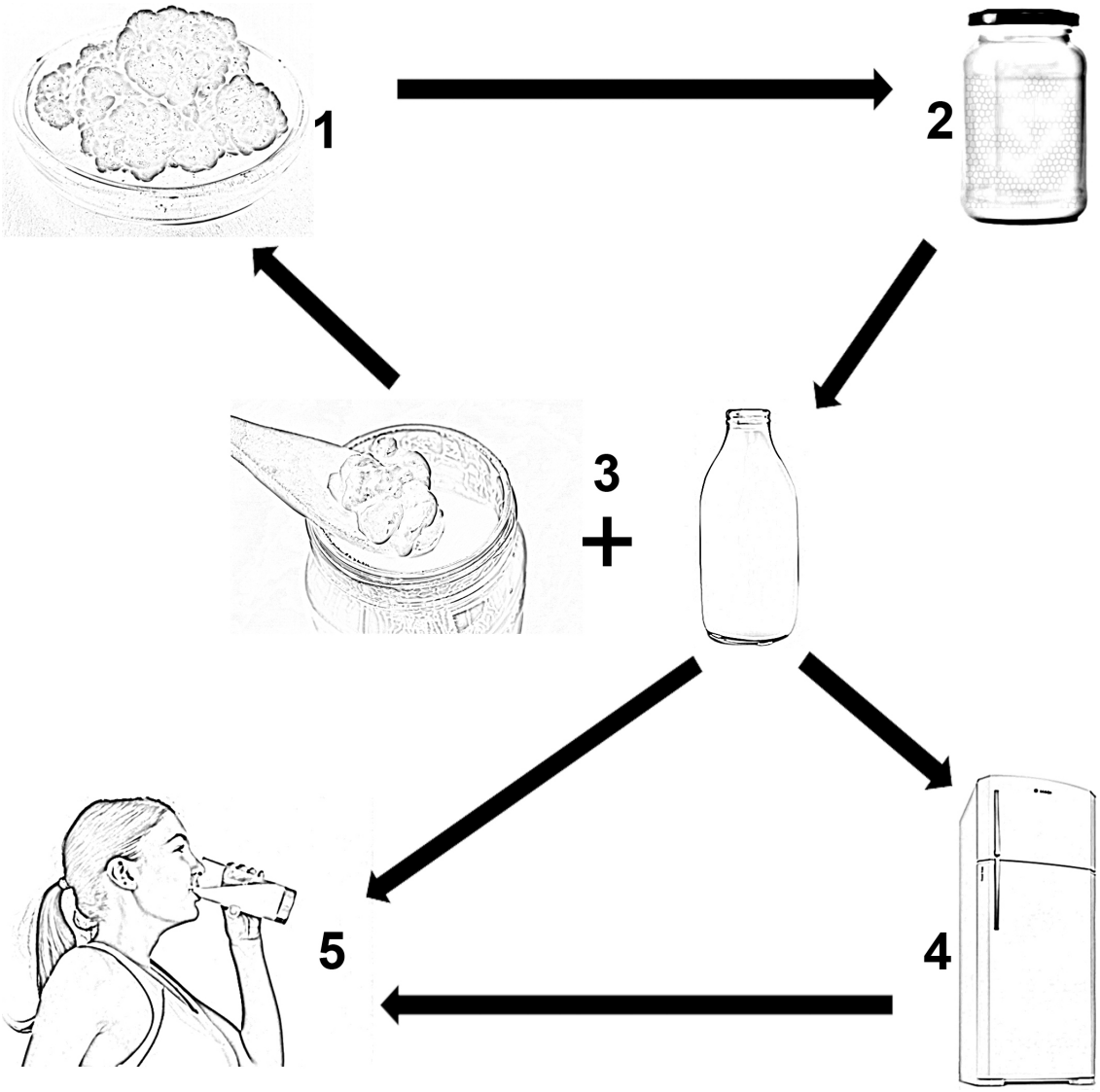


**Fig. 2** – Domestic production of kefir. 1 – Separation of kefir grains. 2 – Addition of milk to the kefir grains in a half-open container at room temperature to ferment for 10 to 24 h. 3 – Filtration and separation of kefir grains. Possible addition of the kefir grains to fresh milk to start a new fermentation. The kefir is adequate for consumption. 4 – The kefir can be refrigerated (4°C). 5 – The kefir is safe and ready to drink.

### 5.6. Nutritional composition of kefir

The nutritional composition of kefir varies widely and is influenced by the concentration of lipids in milk, the origin of the grains used, and the time/temperature of fermentation, as well as the storage conditions. When ready for consumption, the drink contains lactic, formic, propionic, and succinic acids, $CO_2$, ethanol, aldehydes, traces of

acetone and isoamyl alcohol, and a variety of folates [28]. The pH of kefir varies between 4.2 and 4.6, ethanol content between 0.5 and 2.0% (v/v), lactic acid between 0.8 and 1.0% (w/v), and $CO_2$ between 0.08 and 0.2 % (v/v) [33].

Regarding the chemical composition, moisture is the predominant constituent (86.3%), followed by sugars, protein, fat, and ash [34]. During fermentation, proteins become easily digestible due to the action of the acid coagulation and proteolysis. Kefir shows a similar profile of amino acids as the milk used as the culture medium [35]. The levels of ammonia, serine, lysine, alanine, threonine [14], tryptophan, valine, lysine, methionine, phenylalanine, and isoleucine are higher in kefir compared with unfermented milk [34].

During the fermentation, lactose from milk is degraded to acid, which causes the pH reduction and the increase of the consistency. Approximately 30% of milk lactose is hydrolyzed by β-galactosidase enzyme, turning lactose into glucose and galactose. Bacteria present in kefir convert glucose into lactic acid [35].

The lipid content (mono-, di-, and tri-glycerides, free fatty acids, and steroids) in kefir can vary depending on the type of milk used in the fermentation. In the fermented milk, the presence of free fatty acids contributes to the improvement of digestibility [24].

Kefir is an important dietary source of some minerals and vitamins. Among the minerals, calcium, phosphorus, potassium, magnesium, zinc, copper, manganese, iron, cobalt, and molybdenum are found [34]. During fermentation, pyridoxine, vitamin B12, folic acid, biotin thiamin, and riboflavin increase in concentration [34].

### 5.7. Microbiological composition of kefir

The microbiota present in kefir drink and grains include numerous bacterial species from lactic acid and acetic acid groups, yeasts, and filamentous fungi, which develop complex symbiotic associations [3]. In this relationship, yeasts produce vitamins, amino acids, and other essential growth factors that are important for bacteria. Likewise, the metabolic products of bacteria are used as an energy source for the yeasts. This symbiosis allows the maintenance of stability, so that throughout the fermentation cycle, the microbiological profile of kefir grains and kefir drink remains unaltered despite variations in the quality of the milk, microbial contamination, presence of antibiotics, and other inhibitory substances [25].

The identification of microbiota present in kefir beverage and grains is important since it is directly related to the quality of the probiotic product [36]. Different methodologies have been applied to study the kefir microbiota; however, the classical

approach of culturing microorganisms in nutrient media (universal, selective) and identification of isolated cultures is still the most commonly used and acceptable method [37]. Using culture-independent methods such as metagenomics, the vast number of previously unknown microorganisms in kefir have been characterized [38] .

The microbial diversity of kefir described in the literature varies greatly (**Table 1**). The number of different microbial species is estimated to be more than 60. Witthuhn and co-workers [39] observed that the population of bacteria in kefir may vary from $6.4 \times 10^4$ to $8.5 \times 10^8$ CFU/g and yeasts from $1.5 \times 10^5$ to $3.7 \times 10^8$ CFU/g. After 24 h of fermentation, kefir presented $10^8$ CFU/mL *Lactobacillus*, $10^5$ CFU/mL *Lactococcus*, $10^6$ CFU/mL yeasts, and $10^6$ CFU/mL acetic acid bacteria [40].

According to Lopitz-Otsoa *et al.*[2] the microbial composition of kefir grains comprised 65 to 80% *Lactobacillus* and *Lactococcus* and the remaining portion was completed by yeasts. Halle *et al.* [41] found that 80% of lactobacilli belonged to *L. kefiri* and the remaining 20% belonged to *Lactobacillus paracasei* subsp. *paracasei, Lactobacillus acidophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus plantarum*, and *Lactobacillus kefiranofaciens.* Yeast species such as *Saccharomyces cerevisiae, Saccharomyces unisporus, Candida kefyr*, and *Kluyveromyces marxianus* subsp. *Marxianus* are present in kefir drink and grains in greater numbers

**Table 1**. Species found in the microbiota of kefir and its grains.

| **Species** | **References** |
|---|---|
| Lactobacilli | |
| *Lactobacillus acidophilus* | Ângulo *et al*.[85]; Santos *et al*.[86] |
| *Lactobacillus brevis* | Simova *et al*.[29]; Mobili *et al*.[87] |
| *Lactobacillus casei* | Simova *et al*.[29]; Zhou *et al.* [88] |
| *Lactobacillus crispatus* | Garbers *et al.* [89]; Leite *et al.* [90] |
| *Lactobacillus delbrueckii* | Simova *et al.* [29]; Santos *et al.* [86] |
| *Lactobacillus fermentum* | Ângulo *et al*.[85]; Witthuhn *et al.* [91] |
| *Lactobacillus fructivorans* | Yoshida and Toyoshima [92] |
| *Lactobacillus gasseri* | Ângulo et al.[85]; Molska *et al.* [93]; |
| *Lactobacillus helveticus* | Simova *et al.* [94]; Valasaki *et al.* [95] |
| *Lactobacillus hilgardii* | Yoshida and Toyoshima [92] |
| *Lactobacillus kefir* | Santos *et al.* [86]; Unsal [96] |

| | |
|---|---|
| *Lactobacillus kefiranofaciens* | Wang *et al.* [97]; Leite *et al.* [90] |
| *Lactobacillus kefirgranum* | Takizawa *et al.* [98] |
| *Lactobacillus mesenteroides,* | Garbers *et al.* [89] |
| *Lactobacillus paracasei* | Santos *et al.* [86]; Magalhães *et al.* [99] |
| *Lactobacillus parakefiri* | Garrote *et al.* [100]; Leite *et al.* [90] |
| *Lactobacillus plantarum* | Santos *et al.* [86]; Gao *et al.* [101] |
| *Lactobacillus rhamnosus* | Angulo *et al.* [85] |
| *Lactobacillus viridescens* | Molska *et al.* [93]; Angulo *et al.* [85] |
| *Lactococci* | |
| *Lactococcus lactis* subsp. *lactis* | Yuksekdag *et al.* [102];Witthuhn *et al.* [91] |
| *Lactococcus lactis* subsp. *cremoris* | Yuksekdag *et al.* [102]; Mainville *et al.* [103] |
| *Lactococcus lactis* subsp. *lactis biovar. diacetylactis* | Garrote *et al.* [100] |
| *Streptococci* | |
| *Streptococcus cremoris* | Ergullu and Ucuncu [104] |
| *Streptococcus durans* | Yuksekdag *et al.* [102] |
| *Streptococcus faecalis* | Ergullu and Ucuncu [104] |
| *Streptococcus thermophilus* | Yuksekdag *et al.* [102]; Simova *et al.* [29] |
| *Leuconostoc mesenteroides* | Garrote *et al.* [100] |
| *Acetic Acid Bacteria* | |
| *Acetobacter aceti* | Koroleva [105]; Angulo *et al.* [85] |
| *Acetobacter sp* | Garrote *et al.* [100] |
| *Acetobacter pasteurianus* | Ottogalli *et al.* [106] |
| *Other bacteria* | |
| *Bacillus sp* | Angulo *et al.* [85] |
| *Bacillus subtilis* | Ottogalli *et al.* [106] |
| *Escherichia coli* | Angulo *et al.* [85] |

| | |
|---|---|
| *Micrococcus sp* | Angulo *et al.* [85] |
| *Bifidobacterium spp* | Leite *et al.* [90] |
| *Yeast* | |
| *Brettanomyces anomalus* | Wyder and Puhan [107] |
| *Candida albicans* | Angulo *et al.* [85]; |
| *Candida friedricchi* | Angulo *et al.* [85]; Wyder [108] |
| *Candida holmii* | Angulo *et al.* [85]; Kumura *et al.* [109] |
| *Candida inconspicua* | Simova *et al.* [29] |
| *Candida kefir* | Berruga *et al.* [110]; Wyder [108] |
| *Candida lambica* | Engel *et al.* [111] |
| *Candida maris* | Simova *et al.* [29] |
| *Candida pseudotropicalis* | Ottogalli *et al.* [106] |
| *Candida tannotelerans* | Dousset and Caillet [112] |
| *Candida tenuis* | Ottogalli *et al.* [106] |
| *Candida valida* | Dousset and Caillet [112] |
| *Issatchenkia occidientalis* | Latorre-Garcia *et al.* [113]; Wyder [108] |
| *Kluyveromyces lactis* | Wyder [108]; Latorre-Garcia *et al.* [113] |
| *Kluyveromyces marxianus* | Garrote *et al.* [100]; Wang *et al.* [97] |
| *Pichia fermentas* | Angulo *et al.* [85]; Wang *et al.* [97] |
| *Saccharomyces cerevisiae* | Simova *et al.* [29]; Zhou *et al.* [88] |
| *Saccharomyces delbruecki* | Pintado *et al.* [114]; Engel *et al.* [111] |
| *Saccharomyces exiguus* | Koroleva [105]; Latorre-Garcia *et al.* [113] |
| *Saccharomyces humaticus* | Latorre-Garcia *et al.* [113] |
| *Saccharomyces turicensis* | Wyder [108]; Wang *et al.* [97] |
| *Saccharomyces unisporus* | Wyder [108]; Latorre-Garcia *et al.* [113] |
| *Torulopsis holmii* | Angulo *et al.* [85]; Wyder [108] |
| *Torulospora delbrueckii* | Angulo *et al.* [85]; Wyder [108] |
| *Zygosaccharomyces florentinus* | Angulo *et al.* [85]; Wyder [108] |
| *Weissella* | Gao *et al.* [38] |
| *Yarrownia lipolytica* | Angulo *et al.* [85]; Wyder [108] |

## 5.8. Physiological effects of kefir

A schematic diagram of the beneficial effects of kefir on human physiology and health is shown in **Fig. 3.** The main findings from recent *in vitro* and *in vivo* studies on animal models and humans are presented in **Table 2**.

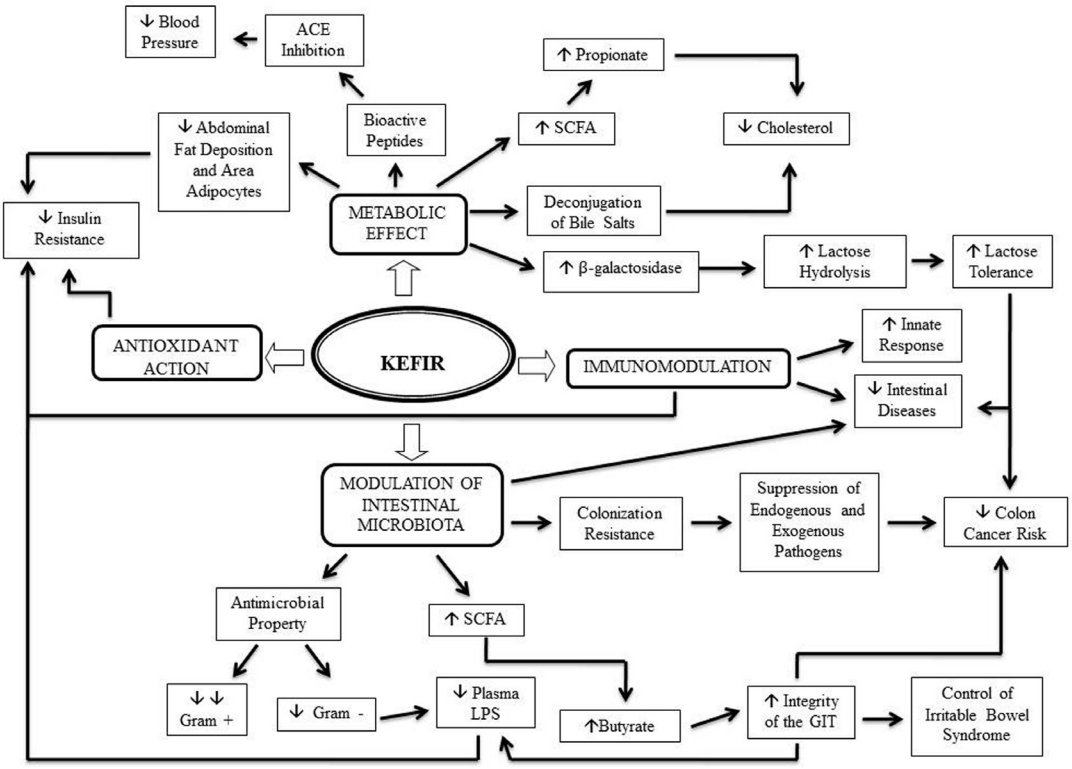


**Fig. 3** – Schematic diagram of the beneficial physiological effects of kefir on human health. LPS: lipopolysaccharide; GIT: gastrointestinal tract; SCFA: short-chain fatty acid; ACE: angiotensin-converting enzyme.

### (1) Antimicrobial properties of kefir

Studies of the early twentieth century observed that the positive effect on life expectancy of regular consumption of yogurt containing lactic acid-producing microorganisms was due to the existing competition between lactic acid bacteria (LAB) and harmful pathogens. Since then, antifungal and antibacterial activities of probiotics like kefir have been extensively studied [(2)].

Antibacterial properties of kefir are related to a combination of several factors, including competition for available nutrients and the inherent action of organic acids, hydrogen peroxide, acetaldehyde, $CO_2$, and bacteriocins produced during the fermentation process [(42)]. These substances also exhibit some effects similar to those of nutraceuticals, preventing gastrointestinal disorders and vaginal infections [(43)].

Kefir exerts bactericidal effects on Gram-negative bacteria; however, it is more potent against Gram-positive bacteria [44]. This antagonistic action has been observed against the bacteria *Salmonella, Helicobacter* [45], *Shigella, Staphylococcus* [11; 45], *Escherichia coli, Enterobacter aerogenes, Proteus vulgaris, Bacillus subtilis, Micrococcus luteus* [46], *Listeria monocytogenes, Streptococcus pyogenes*, and also against the yeast *Candida albicans* [11].

Silva *et al.* [47] reported antimicrobial activity of kefir against *C. albicans, E. coli, Staphylococcus aureus, Salmonella typhi,* and *Shigella sonnei*. Ulusoy *et al.* [48] observed that kefir produced from lyophilized commercial grain (PROBAT KC3, Danisco, Denmark) demonstrated antibacterial effect against S. aureus, Bacillus cereus, Salmonella enteritidis, L. monocytogenes, and E. coli. The results were comparable to the antibacterial action of ampicillin and gentamicin.

Kefir grains have been found to have higher antibacterial activity than kefir drink. This is manifested especially against Gram-positive cocci, including staphylococci and Gram-positive bacilli [1]. The antifungal and antibacterial activities may explain the wide use of kefir in the treatment of infectious diseases and tumor development [49].

According to Brialy and co-workers [50], fresh kefir presented an intrinsic inhibitory potential against *S. aureus*, *Kluyveromyces lactis*, and *E. coli*. However, this effect was not verified against *S. cerevisiae* and *C. albicans*. Kefir has been found to lose its intrinsic inhibitory effect after lyophilization and constitution in distilled water or milk.

In an *in vitro* study, Ismaiel and colleagues [51] tested the antimicrobial activity of kefir grains and kefir suspension against several species of bacteria and fungi and observed higher inhibitory action against *Streptococcus faecalis* and *Fusarium graminearum*. The concentration of kefir from 7% to 10% w/w was able to inhibit *Aspergillus flavus* sporulation, demonstrating the antifungal properties of kefir against filamentous fungi due to the inhibitory effect of kefir on aflatoxin B1.

Kefir was able to increase the population of LAB and reduce the levels of *Enterobacteriaceae* and *Clostridium* in the intestinal mucosa of mice [52]. Oral administration of milk kefir or soy kefir in mice over a period of 28 days was able to significantly increase the *Lactobacillus* and *Bifidobacterium* while reducing *Clostridium perfringens* in animal feces [53].

**(2) Hypocholesterolemic action of kefir**

The consumption of fermented dairy products has been proposed as a strategy to reduce levels of circulating cholesterol. Taylor and Williams [13] observed that the consumption of fermented dairy products was able to lower cholesterol levels in 8 of the 12 clinical trials. Several biochemical mechanisms are proposed to justify these findings:

- The LAB inhibit the absorption of exogenous cholesterol in the intestine due to binding and incorporation of cholesterol by the bacterial cells. The high count of LAB present in kefir may directly or indirectly reduce cholesterol by up to 33% [54]. Vujicic *et al.* [55] verified that after 24 h of fermentation, kefir was able to absorb 28 to 65% of the cholesterol present in the culture medium.
- Probiotic bacteria increase the production of short-chain fatty acids (SCFA). Among the different SCFA produced, propionate reduces production of cholesterol by inhibiting hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase enzyme activity. Therefore, plasma cholesterol is redistributed to the liver, increasing the synthesis and secretion of bile acids, since it stimulates the activity of the 7α-hydrolase enzyme. Moreover, propionate inhibits intestinal expression of genes involved in biosynthesis of cholesterol [56].
- Another pathway is by deconjugation of bile acids, which may be increased in the large intestine, caused by the bile salt hydrolase (BSH) enzyme. The BSH enzyme catalyzes the hydrolysis of glycine and/or taurine conjugated to the bile salts in residual amino acids and free bile salts, increasing excretion. Increased synthesis of bile acids reduces cholesterol levels in the circulatory system [1].

Some studies demonstrated the hypocholesterolemic activity of kefir [17; 57]. Hamsters fed a hypercholesterolemic diet associated with freeze-dried kefir (milk or soy) showed a significant reduction in triglyceride levels and atherogenic index [58]. In this study, the effects were partially related to increased fecal excretion of neutral sterols and bile acids. *L. plantarum* MA2 isolated from kefir grains originating from Tibet was effective in reducing cholesterol and triglycerides levels in plasma and liver and it increased fecal excretion of cholesterol and triglycerides in mice fed a high fat diet [59].

Animals that consumed hyperlipidemic diets associated with kefiran showed a reduction in total cholesterol, low-*density* lipoprotein cholesterol *(*LDL-c), and triglycerides in serum, as well as cholesterol and triglycerides in liver compared with controls [17]. Uchida and co-workers [60] evaluated the anti-atherogenic effect of kefiran in rabbits fed a high cholesterol diet and observed lower atherosclerotic lesion in the

abdominal aorta and lower levels of hepatic cholesterol and lipid peroxidation in the animals fed kefiran.

The consumption of kefir (0.5 L/day) by hypercholesterolemic adult men for 4 weeks did not affect circulating levels of total cholesterol, high-lipoprotein cholesterol (HDL-c), LDL-c, or triglycerides; however, it increased the SCFA level in their feces [61]. Such conflicting results may be due to the different experimental protocols used, origin of the grains, and fermentation conditions of the kefir.

**(3) Anti-inflammatory properties of kefir**

The anti-inflammatory effects of probiotics may be due to direct modulatory action on microbiota in the gastrointestinal tract (GIT) or an indirect effect on the immune system through mediators of inflammation, such as cytokines [62].

The anti-inflammatory potential of kefir was evaluated in an animal model of asthma, sensitized with ovalbumin. The administration of kefir (50 mg/kg) was found to significantly inhibit the total number of inflammatory cells and eosinophils in the bronchoalveolar fluids. In addition, kefir administration decreased elevated interleukin 4 (IL-4), interleukin 13 (IL-13), and immunoglobulin E (IgE) to a normal level [12]. Thus, kefir has therapeutic potential for the treatment of allergic bronchial asthma.

Rodrigues and co-workers [11] evaluated the anti-inflammatory action of kefir in rats using a protocol of edema and granuloma induction. In this study, water kefir, milk kefir, and kefiran extract inhibited the inflammatory process by 41, 44, and 34%, respectively. The treatments also significantly reduced edema in the animals. The results demonstrate the presence of anti-inflammatory compounds in the symbiotic cultures of kefir.

Kefir has been found to reduce intestinal permeability against foodborne antigens. Liu *et al*. [53] evaluated the presence of immunoglobulin (IgE and IgG) in the serum of mice that were treated with ovalbumin and consumed milk and soy kefir for 28 days. Animals fed kefir showed lower responses to IgE and IgG, suggesting the potential of this beverage in the prevention of food allergy and in the improvement of mucosal resistance against pathogen infection. According to Thourex *et al*. [63] addition of kefir to the diet can affect intestinal mucosal and systemic immune responses in rodents but this effect is more pronounced in young rats.

**(4) Effect on immune system**

Fermented products rich in LAB have potential for improving the immune response in animal models and in humans [43; 64]. *In vitro* studies have demonstrated the immunomodulatory capacity of LAB isolated from kefir grains, suggesting a strong influence on the secretion of proinflammatory cytokines such as IL-6 and TNF-α via TLR-2 [65]. Immunomodulatory properties of kefir may result from direct action of the microbiota or may be indirect, through different biogenic compounds produced during the fermentation process [66].

Bioactive peptides are produced during milk fermentation by the microbiota present in kefir. Such peptides are able to activate macrophages, increase phagocytosis, nitric oxide, and cytokines, and stimulate the secretion of IgG and IgA by B lymphocytes in the intestinal lumen [67].

The immunomodulatory capacity of kefir is related to the microbiota present, which beneficially influences intestinal mucosal health. The components of kefir can stimulate the innate immune response and suppress the Th2 immune response. Thus, these components promote cell-mediated immune response against infections and intracellular pathogens [49]. According to Vinderola and co-workers [64], kefir has the ability to induce a more potent immune response in intestinal mucosa, which helps to maintain intestinal homeostasis. The supply of kefir to cholera toxin-sensitized mice for a period of 28 days was able to enhance mucosal immune response (IgA) [63].

**(5) Antioxidative activity of kefir**

The antioxidative activity of kefir is unclear so far. Harmful biological effects of reactive oxygen species *in vivo* are controlled by a broad spectrum of antioxidant defense mechanisms. According to Guven and colleagues [28], kefir exerts a higher antioxidant effect than vitamin E in a toxicity test with carbon tetrachloride ($CCl_4$) in rodents.

Ozcan *et al*. [68] evaluated the effect of kefir supplementation in rodents induced to oxidative stress by the use of lead. After 6 weeks of treatment, the consumption of kefir increased glutathione peroxidase and reduced malondialdehyde to levels comparable to those of the non-induced group. The results support the hypothesis that kefir is a potential tool in the control of oxidative stress.

Liu and co-workers [69] evaluated the antioxidative activity of kefir prepared from goat and cow milk. The group reported the great binding ability of kefir to the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and superoxide radicals, in addition to inhibition of linoleic acid peroxidation. The antioxidative activity of kefir can reduce

DNA damage, which explains its anticancer potential [70]. The presented data suggest the great potential of kefir as an important natural antioxidant in the human diet.

**(6) Anticarcinogenic action of kefir**

The anticarcinogenic action of fermented milk can be attributed to the prevention and suppression of the early stages of tumor formation. A study performed by Larsson and collaborators [71] suggests that high consumption of fermented milk, milk curd, and yogurt may reduce the risk of developing bladder cancer. The reduction of breast cancer risk can be attributed to the presence of bioactive components such as specific proteins and peptides in kefir [43]. The intestinal microbiota and immune system activation play an important role in the modulation of carcinogenesis. To understand the mechanisms of action and antimutagenic properties of kefir, researchers evaluated the profile of kefir microorganisms that originated from Mongolia, and isolated the strains of *Streptococcus lactis*, *Streptococcus cremoris*, *S. faecalis*, *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Leuconostoc dextranicum*. Using a binding assay in which the bacteria were incubated with mutagenic amino acid pyrolysates, it was observed that all bacteria isolated from kefir had a remarkable ability of binding to mutagens (>98.5%). The authors concluded that similarly to the bacteria isolated from yogurt, the consumption of fermented dairy products had a negative correlation with the risk for colon cancer development [72].

Oral administration of milk and soy kefir in mice inoculated with Sarcoma 180 cells resulted in inhibition of 64.8% and 70.9% of tumor growth, respectively, compared with administration of unfermented milk. Furthermore, kefir was able to induce cell lysis by apoptosis and increase levels of IgA in the intestinal mucosa of animals after 30 days of consumption. These data suggest that kefir is a promising probiotic in cancer prevention and increased mucosal resistance to gastrointestinal infection [49].

The antimutagenic activity of milk, yogurt, and kefir were compared using the Ames test. Kefir showed a significant reduction in the mutagenicity induced by methyl methanesulfonate, sodium azide, and aflatoxin B1, while yogurt and milk reduced the mutagenicity to a lesser degree. In kefir, higher levels of conjugated linoleic acids (CLA) isomers and butyric, palmitic, palmitoleic, and oleic acids were found in relation to milk and yogurt, factors that may have contributed to the reported outcomes [73].

**(7) Healing action of kefir**

Recent studies have explored the beneficial effects of probiotics far beyond the intestine. Some of these novel benefits include healthier skin, improvement of eczema, atopic dermatitis, and burns, healing of scars, and rejuvenation [74].

The healing properties of kefir were tested in an animal model with experimental burn and contamination with *Pseudomonas aeruginosa*. Kefir grains and gels prepared with kefir culture after 24, 48, and 96 h of incubation were evaluated. After 2 weeks of treatment, wound area and percentage of inflammation were reduced in animals treated with kefir grains and gel compared with those treated with silver sulfadiazine cream, used for topical treatment of burns of second and third degrees. In addition, the percentage of epithelialization and healing in animals treated with kefir was also better. The authors concluded that treatment with kefir gel was effective in improving outcomes following a severe burn compared with conventional treatment [75].

**(8) Anti-hypertensive action of kefir**

Some evidence indicates that probiotic bacteria or their fermented products play an important role in controlling blood pressure; the antihypertensive effects have been observed in clinical studies and with animals [76], although the data are limited and controversial.

According to Maeda *et al*.[17], rats that consumed kefiran showed a reduction in blood pressure due to inhibition of angiotensin-converting enzyme (ACE) activity. The ACE inhibitory peptides can be released from milk by microbial activity during fermentation.

The inhibitory peptides of ACE can be derived from a variety of fermented products including cheese, fermented milk, soy milk, yogurt, and kefir [62]. Quiros and colleagues [77] found that the ACE inhibitory capacity of kefir produced from goat milk was related to the formation of low molecular weight peptides from casein fermentation.

**(9) Effect of kefir on lactose intolerance**

Milk and dairy products contain high concentrations of the lactose (glucose and galactose) disaccharide. Intestinal absorption of lactose requires hydrolysis of the disaccharide and its subsequent absorption in the small intestinal mucosa. A significant part of the world population demonstrates limitations in the digestion of lactose due to insufficient activity of intestinal β-galactosidase [78]. This enzyme, naturally present in kefir grains, reduces lactose content of the milk during fermentation, which in turn makes the final product suitable for people with lactose intolerance [43]. It is noteworthy

to mention that fermented products are characterized by a lower intestinal transit time, which helps in lactose digestion [79].

**(10) Effect of kefir on blood glucose**

Hadisaputro *et al*. [19] evaluated the effect of kefir consumption for 30 days in controlling glycemia and immune response in Wistar rats induced with diabetes mellitus by administration of streptozotocin. Kefir supplementation was able to reduce plasma glucose and pro-inflammatory cytokines (IL-1, IL-6, and TNF-α) compared with the control group. In another study, Kwon et al. [80] observed that kefir produced from soy milk associated with the *Rhodiola* extract had a great anti-diabetic potential due to its ability to mobilize phenolic compounds that alter postprandial hyperglycemia and control type II diabetes.

**(11) Kefir consumption and gut health**

The beneficial effects of probiotics on gut health may be attributed to their proteolytic activity in GIT. In the GIT, probiotics contribute to the hydrolysis of enteral antigens, reduces secretion of inflammatory mediators, improves the integrity of the intestinal mucosa, normalizes intestinal permeability, and increases IgA production in the mucosa. Furthermore, healthy intestinal microbiota may decrease the activity of fecal enzymes and toxins, which are important factors in colon cancer prevention [81]. In addition, probiotics have a strong potential to control enteropathogens [82].

There is a hypothesis that both kefiran and the bacteria found in kefir can affect intestinal penetration of food allergens and the resulting food sensitization. The consumption of kefir by rodents before exposure to radiation may significantly influence the rate of apoptosis in the colonic mucosa. Finally, kefir can also stimulate the mucosal immune system, wherein the immunomodulatory effect is related to bacterial cell wall components [27].

The presence of yeast populations such as *S. cerevisiae, Kluyveromyces lodderae*, *K. marxianus*, and *Candida humilis* in kefir has an important effect on intestinal colonization by microorganisms [2]. Marquina *et al*. [52] observed that oral administration of kefir to rodents was able to alter the microbiological profile of the small and large intestines of animals; the levels of LAB were increased and *Enterobacteriaceae* and *Clostridium* were reduced. Yang *et al.* [83] investigated the effect of ingestion of fermented milk containing *Bifidobacterium lactis* associated with yogurt yeasts in 135 Chinese adult women suffering from constipation. The study found

that after 2 weeks of probiotic consumption, the women showed a significant improvement in bowel function

Figler *et al*. [81] evaluated the fecal contents of healthy adults consuming traditional Russian kefir (0.5 L/day) for 4 weeks, with and without addition of lactic acid probiotic bacteria. The total number of bacteria present in the fecal material increased in both groups, which demonstrates the probiotic activity of kefir. Nevertheless, human studies are still limited in this area.

**Table 2**: Health benefits of milk kefir

| Effects | Study design | Objectives | Main Results | Reference |
|---|---|---|---|---|
| Antimicrobial | Randomized study<br><br>*In vitro* | To evaluate the effect of the initial inoculum and the fermentation time on the antibacterial activity of kefir grains | 5% inoculum with 24 h fermentation was the optimal condition for the highest antibacterial activity.<br>The effect depended on the origin of the kefir grains and the number of lactococci present.<br>*Escherichia coli* and *Staphylococcus aureus* were less sensitive to kefir supernatant than *Klebsiella pneumoniae* and *Salmonella enterica* | Hristova et al. (2012) |
| | Case-Control Study<br><br>*In vitro* | To investigate the antimicrobial activity of kefir (fermented 24/48h, fresh or after 7 days) against *B.subtilis spp. spizizenii, S. aureus, E. faecalis, E. coli, S. enteritidis, P. aeruginosa, C. albicans* in comparison with ampicillin and neomycin. | The antimicrobial activity of kefir was higher than the both tested antibiotics for *E. coli*, *E. faecalis* and *S. enteritidis*.<br>The tested products exhibited no activity against *P. aeruginosa* and *C. albicans*. | Chifiriuc et al. (2011) |
| Healing Activity | Case-Control Study<br><br>Wistar rats | To evaluate the effect of saline solution, neomycin–clostebol emulsion and kefir gel on a shaved dorsal area wound infected with *S. aureus.* | Both kefir and kefiran showed the highest activity against *Streptococcus pyogenes.*<br>Cicatrizing experiments using 70 % kefir gel had a protective effect on skin connective tissue and the 7 days of treatment enhanced the wound healing | Rodrigues et al. (2005) |

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|---|---|---|---|---|
| | | | compared to neomycin–clostebol emulsion. | |
| Immune System | Prospective and self-controlled study<br><br>18 healthy volunteers | To study the effect of kefir consumption (200 mL /day) for six weeks. | ↓ serum IL-8 and TNF-α level due to kefir use.<br>↓ serum IL-5 levels at 3rd week of consumption.<br>Hemoglobin, creatinine and ALT levels did not change due to kefir consumption. | Adiloğlu et al. (2013) |
| | Case-Control Study<br><br>BALB/c mice | To investigate the effect of oral administration of kefiran (300 mg/L) ad libitum in drinking water for 2 or 7 consecutive days | Kefiran treatment:<br>↑ IgA in lamina propria after 2 and 7 days.<br>↑ B220+/MHCII in mesenteric lymph nodes (2 days).<br>↑ B220+/MHCII in Peyer's patches (7 days).<br>↑ macrophages in lamina propria and peritoneal cavity (2 and 7 days). | Medrano et al. (2011) |

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|---|---|---|---|
| *In vivo* and *in vitro* study | To evaluate the effects of *Lactobacillus kefiranofaciens* M1 isolated from Taiwanese milk kefir grain on intestinal epithelial cells *in vitro* and on dextran sodium sulfate (DSS)-induced colitis *in vivo.* | Effect of *Lb. kefiranofaciens* M1 *in vitro*:<br>↑ epithelial barrier function by increasing the transepithelial electrical resistance (TEER).<br>↑ chemokine CCL-20 at both the apical and basolateral sites.<br>Effect of *Lb. kefiranofaciens* M1 *in vivo*:<br>↓ bleeding score and colon length shortening.<br>↓ proinflammatory cytokines.<br>↑ antiinflammatory cytokine (IL-10). | Chen et al (2012) |
| Case-Control Study<br><br>BALB/c mice | To investigate the oral administration of efir suspension (200 mL/day) on cytokine expression in blood serum and intestinal fluid, for 2, 5 or 7 consecutive days. | ↑ IgA cells in the small and large intestine.<br>↑ IL-4, IL-10 and IL-6 cells in the small intestine.<br>In the blood serum ↑ IL-4, IL-10 and IFNγ for the period of 7 days and ↑TNF-α for the period of 2 days of kefir administration, compared with control mice, were observed. | Vinderola et al. (2006) |
| Case-Control Study<br><br>BALB/c mice | To evaluate the oral administration of commercial kefir and pasteurized kefir with different dilutions, for 2, 5 or 7 consecutive days | Th1 response was controlled by Th2 cytokines induced by kefir feeding.<br>Pasteurized kefir was able to induce both Th2 and Th1 responses. | Vinderola et al. (2005) |

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|---|---|---|---|---|
| Anticarcinogenic Action | *In vitro* study<br><br>Mammary cancer cells (MCF-7) and normal human mammary epithelial cells (HMECs) | To study the antiproliferative effects of kefir extracts, yogurt, and pasteurized cow's milk at the doses of 0.31 %, 0.63 %, 1.25 %, 2.5 %, 5 %, and 10 % (vol/vol) for 6 days. | Kefir ↓ MCF-7 cell growth in a dose-dependent manner.<br>Kefir at a dose 0.63 %, ↓ MCF-7 cell numbers by 29 %.<br>Kefir at a dose 2.5 %, ↓ MCF-7 cell numbers by 56 %.<br>Yogurt at a dose 2.5 %, ↓ MCF-7 cell proliferation by 14 %. No antiproliferative effects of kefir were observed in the HMEC. | Chen et al. (2007) |
| | Case-Control Study<br><br>BALB/c mice | To evaluate the oral administration of kefir and a kefir cell-free fraction (KF) in mice induced with breast tumor cells for 2 days | ↑ IgA(+) cells in the mammary gland KF group.<br>↑ CD4+ cells increased apoptotic cells.<br>↓ Bcl-2(+) cells. | de Moreno de LeBlanc et al (2007) |
| | Case-Control Study<br><br>BALB/c mice | To investigate the oral administration of cow milk and soy milk kefirs in mice inoculated with sarcoma 180 tumor cells, for 30 days | Cow milk kefir resulted in 64.8 % inhibition of tumor growth.<br>Soy milk kefir resulted in 70.9 % inhibition of tumor growth.<br>Two kefir types induced apoptotic tumor cell lysis.<br>Two kefir types ↑ IgA in small intestine. | Liu et al. (2002) |

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|---|---|---|---|---|
| Lactose Intolerance | Randomized block design<br><br>15 healthy adults with lactose maldigestion | To evaluate the effect of milk, plain yogurt, plain kefir, flavored yogurt and flavored kefir (all with 20g lactose portions) in individuals. | Kefir consumption reduced the severity of flatulence by 71 %.<br>Lesser lactose contents and higher β-galactosidase activity was observed in kefir. | Hertzler and Clancy (2003) |
| Cholesterol | Case-Control Study<br><br>Sprague Dawley rats | To study the effect of a cholesterol-enriched experimental diet supplemented with lyophilized *Lactobacillus plantarum* MA2 isolated from Chinese traditional Tibetan kefir ($10^{11}$ cells/day/rat) in rats | Animals supplemented with lyophilized *Lactobacillus plantarum* MA2 presented:<br>In serum, ↓ TC, LDL-c and TAG level.<br>In liver, ↓TC and TAG.<br>In feces, ↑ TC, TAG, LAB and bifidobacteria. | Wang et al (2009) |
| | Case-Control Study<br><br>Sprague Dawley rats | To evaluate the effect of a cholesterol-enriched experimental diet supplemented with *Lactobacillus plantarum* strains (Lp09 and Lp45) obtained from kefir grains in rats for 4 wk. | In the LAB-treated rats compared to rats fed with a high-cholesterol diet without LAB supplementation:<br>In serum, ↓ TC, TAG and TAG.<br>In liver, ↓TC and TAG.<br>In feces, ↑ TC and bile acid levels. | Huang et al. (2013) |
| | Case-Control Study<br><br>Sprague Dawley rats | To investigate the effect of *Lactobacillus plantarum* Lp27 isolated from Tibetan kefir grains on hypercholesterolemic rats at a dose | The Lp27 feeding:<br>In serum ↓TAG, TC, LDL-c,<br>In liver, ↓ TAG and TC. | Huang et al. (2013) |

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|---|---|---|---|---|
| | | of $10^9$ CFU/day for 4 wk | In duodenum and jejunum, ↓ NPC1L1. | |
| | Case-Control Study<br><br>Sprague Dawley rats | To study the effect of three lactobacilli strains isolated from kefir grains (*L. acidophilus LA15, L. plantarum B23, L. kefiri D17*) in rats fed with high-cholesterol diet for 4 weeks. | In animals, *Lactobacillus* treated-rats compared to rats fed with a high-cholesterol diet without supplementation:<br>In serum, ↓ TC, TAG and LDL-c.<br>In feces, ↑ TC and TAG.<br>↑↑ LAB treated groups compared to the control groups.<br>The 3 tested strains were detected in the rat small intestine, colon and feces during the feeding trial. | Zheng et al, 2013 |
| Anti-inflammatory | Case-Control Study<br>BALB/c mice | To evaluate the effect of kefir in an allergy induced-mouse and to assess whether kefir can prevent cardiomyocytes inflammation and apoptosis | In animals, the allergy group treated with kefir presented:<br>Reduced or retarded expression of p-NFkB, TNF-α, p38 and Bad protein. | Wang et al, (2012) |

↑= increase; ↓= decrease

### 5.9. Kefir in food

In Russia, USA, Japan, and Central and Northern Europe, kefir has been used in the control and treatment of many diseases due to its nutritional and therapeutic aspects. Recently, a new formulation of kefir with the addition of enzymes such as lipase or α-amylase to prevent and control obesity was patented in Japan. In the former Soviet Union countries, the consumption of kefir has been informally recommended for healthy people to reduce the risk of chronic diseases and also for patients with gastrointestinal and metabolic disorders, hypertension, ischemic heart disease, weight control, and allergies [(81)].

The industrial production of kefir is large in Germany, Austria, France, Luxembourg, Norway, Switzerland, Czech Republic, Slovakia, Poland, and Israel. The last has rules that define the composition and method of kefir production. In Brazil, the consumption of kefir occurs domestically with spontaneous fermentation of kefir grains in milk, without control of the time or temperature of fermentation. The consumption and industrial production of kefir on a larger scale are lacking so far

### 5.10. Final considerations

Milk kefir is a functional food with a great potential. Numerous observed physiological effects support the health-promoting benefits of kefir. However, different study designs of clinical trials on kefir cause difficulties in drawing clear conclusions. In addition, it has been recently found that different manufacturing conditions alter the original characteristics of probiotics [(84)], which therefore may influence their physiological effect on health. In this context, more research on kefir is needed, with the focus on the interaction between microbiota and host, and mechanisms of action and detoxification, which may positively affect human and animal well-being.

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# 6. ARTICLE 2: Kefir reduces insulin resistance and inflammatory cytokine expression in animal model of the metabolic syndrome

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**Running title:** Kefir in the management of metabolic syndrome

**Abbreviations**: Metabolic syndrome (MetS); monosodium glutamate (MSG); body mass index (BMI); high-lipoprotein cholesterol (HDL-C); area under curves (AUC), colony forming unit (CFU); aspartate aminotransferase (AST), alanine aminotransferase (ALT); very low-density lipoprotein (VLDL–c) and low-density lipoprotein cholesterol (LDL–c)

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## 6.1. Abstract

**Introdution** Kefir is a viscous, acidic and mildly alcoholic milk beverage produced by the fermentation of milk using kefir grains as a starter culture. There is a growing evidence that kefir can be beneficial in the maintenance of many diseases, such as metabolic syndrome (MetS). The aim of the present study was to evaluate the effect of kefir supplementation in S*pontaneously Hypertensive Rats* (SHR) in which MetS was induced with monosodium glutamate (MSG), and to determine the protective effect on metabolic parameters, inflammatory, oxidative, histology markers and glycemic index control.

**Methods** Twenty male SHR received neonatal intradermal injections of MSG and were divided into two groups. For the negative control, ten male SHR received neonatal intradermal injections of saline solution (0.9 % NaCl solution). The rats were randomly divided into three groups (10 animals in each group): negative control (NC, 1mL saline solution/day), positive control (PC, 1mL saline solution/day) and kefir group (1 mL kefir/day). Feeding was carried out by gavage. Body weight, food intake, obesity markers, arterial blood pressure, biochemical analyses, oral glucose tolerance tests, oxidative stress markers and inflammatory cytokine expression in adipose tissue were assessed. Pancreatic and adipose tissues were evaluated for any histological changes.

**Results** The neonatal administration of MSG affected lipid and glucose metabolism, and structure of pancreatic and adipose tissues. The kefir supplementation was able to reduce plasma triglycerides, liver lipids, liver triglycerides, insulin resistance, fasting glucose, fasting insulin and HOMA-β, thoracic circumference, abdominal circumference, products of lipid oxidation, proinflammatory cytokine expression (IL-1β) and increase anti-inflammatory cytokine (IL-10) compared to PC group.

**Conclusions** The present findings indicate that kefir is safe and could be beneficial in the management of the MetS.

**Keywords***: kefir, metabolic syndrome, insulin resistance, cytokines, oxidative stress, histology.*

## 6.2. Introduction

Kefir is a natural probiotic beverage produced through the fermentation of yeasts and bacteria naturally present in kefir grains. The final product is a viscous, acidic, and mildly alcoholic milk drink. Kefir originates from the Caucasus and Middle East. The production and consumption of kefir has now spread throughout the world, spurred by its long history of beneficial health effects [(1)]. This fermented milk drink which contains a unique mixture of microorganisms can be beneficial in the maintenance of many disorders and diseases [(2)].

According to FAO/WHO 2002, probiotic is a "live microorganism which when administered in adequate amounts confers a health benefit to the host" [(3)]. The suggested mechanisms of probiotic action include the nutritional effects, removal and inactivation of toxic substances, stimulation of enzymes excretion, production of vitamins and antimicrobial agents, and modulation of the immune response [(4)]. Several studies have emphasized the beneficial properties of probiotic kefir, which include the following: antibacterial [(5)], anti-inflammatory [(5; 6)], healing [(7)], antiallergic [(6)], hypocholesterolemic [(8)], control of lactose intolerance [(9)], beneficial to the digestive tract [(10)], antioxidant [(11)], hypotensive [(12)], anticarcinogenic [(13)], control of serum glucose [(14)], and improvement in gut health [(9)]. Evidences suggest that probiotic bacteria intake could be a useful tool in the control of MetS [(15)]. However, studies demonstrating beneficial effect of kefir on MetS are lacking.

MetS represents a cluster of physiological and anthropometric abnormalities characterized by an abnormally elevated glucose level, obesity, hypertension, elevated triglycerides, and low levels of high-density lipoprotein-cholesterol (HDL-c). MetS is a major contributor to the development of type 2 diabetes and other conditions such as gout, hyperuricemia, oxidative stress, mild kidney disease, endothelial dysfunction, and chronic inflammation [(16)].

Obesity, an essential component in MetS, has a strong connection with inflammation, and there is also a positive correlation between adipose tissue and the expression of proinflammatory mediators. One consequence of this inflammatory status is high insulin resistance, since inflammatory cytokines from adipose tissue have been shown to be able to interfere directly in the signaling pathways of insulin [(15)].

Since, obesity is a precursor for MetS, preventing or treating obesity is a crucial factor in the management and control of MetS. However, strategies like exercise and

behavioral modifications require self-control and are difficult to adopt. Similarly, the current pharmacological therapies suffer from drawbacks of adverse side-effects and the high cost of treatment [17]. Hence, in the present scenario, the development of dietary strategies, i.e. designing natural food products with probiotics (such as kefir) that modulate MetS would be a cost-effective approach and without the fear of adverse side effects on health.

The use of SHR associated with MSG is an important animal model for studying MetS [18; 19; 20]. With these in mind, we evaluated the effect of kefir supplementation in MSG-induced MetS in SHR animals. Our aim was to determine the protective effect of kefir on physiological and histological parameters, inflammatory markers, oxidative stress and glycemic index control in animal model.

## 6.3. Experimental methods

*Kefir preparation*

Kefir particles (grains of kefir, obtained from a private household in Viçosa, Brazil) were washed with distilled water and inoculated in integral cow's milk, pasteurized type C (composition: 3.5 % protein, 5 % carbohydrate, 3 % fat, 1.2 mg/mL calcium, 0.6 mg/mL sodium, giving total of 2.56 kJ/mL) and stored at 4 °C. The kefir drink was made by adding kefir grains to fresh milk at 5 % (wt/wt). After incubation at 25-28 °C for 24 hours, kefir grains were separated from the fermented milk, filtered through a plastic sieve and washed before reusing for subsequent fermentations [10; 21]. This process was repeated daily throughout the experiment.

*Animals and Experimental Design*

*Induction of MetS*

Thirty SHR male newborns were obtained from the Central Animal House at the Center of Biological Sciences at the Federal University of Viçosa. For the induction of MetS, 20 two-day-old male rats received intradermal injections of monosodium glutamate (MSG, 4 mg/g body weight, Sigma Co., St. Louis, MO), until they completed the age of six days, which gave a total of five applications, according to Cunha *et al.* [22]. For negative control (NC), 10 two-day-old male rats received intradermal injections of saline solutions (0.9 % NaCl/day) in the same conditions. After weaning, all the animals were housed in

collective cages for 100 days with food and water *add libitum* until the MetS was developed.

*Animal care and kefir supplementation*

The rats with induced MetS were randomly divided into two groups (10 animals in each group): positive control (PC, 1mL 0.9 % sodium chloride solution/day) and kefir group (1mL kefir/day). Ten animals without induced MetS served as negative control (NC, 1mL 0.9 % sodium chloride solution/day). Feeding was carried out by gavage. The animals were housed in individual cages and maintained under standard conditions (12h light/12h dark cycle, 22 ± 1°C room temperature). Standard Nuvilab® food (composition: 19.0 % protein, 56.0 % carbohydrate, 3.5 % fat, 4.5 % cellulose, 5.0 % vitamins and minerals, giving a total of 13.87 kJ/g) and filtered water were provided *ad libitum*. This case-control study lasted 10 weeks. Food consumption was measured daily and body weights were evaluated each week during the entire experimental period. The feed efficiency was calculated as mean body weight gain (g) / total food consumption (g).

After 10 weeks, counted from the beginning of kefir administration by gavage, the rodents were anaesthetized with Halothane (Tanohalo®, Cristália). The tissues were removed for histological analyses and stored at -80 °C for later analysis.

*Biometrics determinations*

After the experimental period, the abdominal circumference (immediately anterior to the forefoot), thoracic circumference (immediately behind the foreleg), and body length (nose–anus length) were evaluated in all rats [23]. The delta abdominal circumference was calculated as the difference between the values obtained after treatment compared to baseline. The body mass index (BMI) was calculated as body weight (g) / length$^2$ (cm$^2$), [24].

*Arterial blood pressure*

To calm down the animals and dilate the tail blood vessels before measurements, the rats were placed inside a warm chamber (about 40°C) for 5 min. Measurements of arterial blood pressure (systolic) were carried out at least five times for each animal and four nearest was considered, to give better reliability of the data. Resting systolic blood pressure

was measured in conscious rats using a tail-cuff pressure meter (LE5001, Panlab, Harvard Apparatus, Wood Dale, IL, USA).

*Biochemical analyses*

Blood samples were centrifuged at 700 x g for 10 minutes in order to obtain serum and heparin and the tubes were stored at -80 °C for later analysis. Total cholesterol, high-density lipoprotein cholesterol (HDL-c), triacylglycerol, C-reactive protein, creatinine, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) and glucose were analyzed in serum through diagnostic test kits (Bioclin®, Diagnostics®, Belo Horizonte, Brazil) and autoanalyzer equipment (COBAS MIRA Plus, Roche Diagnostic Systems, Branchburg, NJ). The very low-density lipoprotein (VLDL–c) and low-density lipoprotein cholesterol (LDL–c) was determined according to the method of Friedwald et al.[25] as follows: VLDL – c = TG/5. The hepatic lipids were extracted from 200 mg of liver with chloroform/methanol (2:1 v/v), according to the method of Folch et al. [26]. Total lipids were estimated by gravimetric analysis. The triglyceride and cholesterol concentrations were determined enzymatically after solubilization in 1 % triton X100 using commercial kits (Bioclin®, Diagnostics®, Belo Horizonte, Brazil). Calcium level was measured by atomic absorption spectrophotometry (Perkin-Elmer model Analyst 100 Atomic Absorption Spectrophotometer).

Plasma insulin and leptin concentrations were measured using a multiple-analyte assay (Adipokine Assay RADPK-81K, Millipore, Bellerica, MA) according to the manufacturer's instructions. Briefly, rat plasma was mixed with anti-rat leptin and anti-rat insulin antibody-coated microspheres. Samples were incubated with a reporter molecule (streptavidin–phycoerythrin conjugate), exposed to laser energy, and analyzed for emitted fluorescence (Luminex 100, Millipore). The values obtained for the samples were compared against a standard curve to determine their concentration. The homeostatic model assessment index (HOMA-IR) and HOMA-β index scores were calculated using fasting serum insulin at the end of the experimental period according to the following formula: HOMA-IR = [insulin (pmol/L) × blood glucose (mmol/L)] / 22.5 [27] and HOMA-β = [20 × insulin (pmol/L)] / [blood glucose (mmol/L) – 3.5], [28].

*Oral Glucose Tolerance Test (OGTT)*

OGTT was performed before and after week 10 of the experimental period. After an overnight fast (12 h), a D-glucose solution (2.0 g/kg body weight) was administered by oral gavage. Blood samples were taken from the tail at 0 (prior to oral glucose dosing), 30, 60, 90, and 120 min after the oral glucose dosing, using an Accu-Chek Advantage Glucometer and Comfort Curve Strips (Roche). Glucose tolerance was calculated during the OGTT by calculating the area under the curve (AUC) for glucose by the trapezoidal method [29].

*Inflammatory cytokine analysis in adipose tissue*

Intra-abdominal adipose tissue samples were obtained from animals in the experimental groups. Adipose tissue (65 mg) was homogenized by ultrasonication on ice in 1 mL of the buffer (10 mM PBS, 0.08 % sodium azide, 1 % BSA, pH 7.4). Then, the extract was centrifuged at 8,000 x g for 5 minutes to pellet the tissue debris and the supernatant was collected for analysis. The inflammatory cytokine concentrations (IL-1β, IL-6, and IL-10) were determined simultaneously using the Rat Plasma Adipokine LINCOplex kit (RECYTMAG-65K-04, Linco Research, Millipore, MA, USA), using Luminex xMAP technology (Lincoplex 200, Linco Research).

*Oxidant Status Markers in the Liver*

- *Antioxidant System Biomarkers*

Each liver sample (100 mg/mL buffer) was homogenized in 50 mM phosphate buffer (pH 7.0) with 1 % Triton X-100. The homogenate was centrifuged at 11.290 g at 4 °C for 10 minutes and the supernatant obtained was used for biochemical analysis. Catalase activity was evaluated according to the method described by Aebi [30] by measuring the rate of decomposition of hydrogen peroxide ($H_2O_2$), and the results are expressed as U catalase/mg protein. The superoxide dismutase activity was determined by a method adapted from Dietrich *et al.* [31], and the results are expressed as U superoxide dismutase/mg protein. The GST enzyme activity was determined according to the protocol of Habig *et al.* [32], and the results are expressed as GST/minutes/mg protein.

- *Peroxidation Biomarkers*

An aliquot of frozen liver (100 mg) was homogenized in 50 mM phosphate buffer (pH 7.0), and the liver concentration of MDA was determined as thiobarbituric acid reactive substances, according to the method of Buege & Aust [(33)]. Lipid hydroperoxides were determined by the ferrous-oxidation of the colorimetric dye xylenol orange as described by Nourooz-Zadeh *et al.* [(34)]. The level of liver protein oxidative damage, indicated by the protein carbonyl content, was measured according to the method of Levine and co-workers [(35)]. The results are expressed as peroxidation biomarkers/mg protein.

*Determination of protein concentration*

Protein concentration in the tissue homogenates was measured by the method of Lowry *et al*. [(36)] using bovine serum albumin as a reference.

*Histological analyses*

Fragments of the pancreas (6 animals in each group) were extracted and fixed in Carson's formalin [(37)]. After dehydration in an increasing gradient of ethanol, tissue was embedded in hydroxyethyl methacrylate resin (Historesin®, Leica). Fragments of the adipose tissues (6 animals in each group) rinsed with saline solution were fixed in 1 % calcium-buffered formalin, dehydrated in an increasing gradient of ethanol, and finally tissues were embedded in paraffin. Both tissues were stained with routine histological hematoxylin and eosin for the histological analysis and 20 images per animal (120 images in each group) were captured by an Olympus BX50 light microscope (Olympus Optical Co. Ltd., Tokyo, Japan) with a video camera (Olympus Q color III™), 20x objective lens, 2048 x 1024 pixel resolution, using the associated analysis software (Q-Capture Pro 6.0).

Adipocyte cell size in the intra-abdominal adipose tissue was measured according to Boqué et al. [(38)]. Briefly, the images of adipose tissue were analyzed (5 000 adipocytes were measured per group) with software Adiposoft (Adiposoft from CIMA, University of Navarra) in order to determine adipocyte tissue area. In pancreatic tissue, the morphometric digital analysis for determining the area of pancreatic islets was performed using the Image Pro-Plus® Software system, version 4.5 (Media Cybernetics).

*Ethics*

The project was approved by the Ethics Committee of the Department of Veterinary Medicine of the Federal University of Viçosa (107/2011) and the experiments were carried out according to the Ethical Principles in Animal Experimentation, adopted by the National Council for the Control of Animal Experimentation.

*Statistical analyses*

Results are presented as mean values with their standard errors. Statistical significance of the difference between groups was assessed by one-way ANOVA followed by Tukey's post hoc multiple comparison test using GraphPad Prism (GraphPad Software Inc, San Diego, CA). For statistical analysis and representation a $p<0.05$ was considered as statistically significant.

## 6.4. Results

*Biometrics determinations and food intake*

The induction of MetS in the rodents was confirmed based on altered MetS markers. Thereafter, kefir was provided for 10 weeks to evaluate how the components present in the beverage affect the metabolism of animals. The weekly evaluation of animal growth showed lower average weight in animals with induced MetS (PC and Kefir) from the second to tenth experimental week, when compared to healthy animals (NC, $p < 0.0001$). Only in the fifth week, the animals supplemented with kefir showed lower body weight when compared to NC and PC ($p < 0.0001$, **Fig. 1A**). The food intake and energy intake of the animals that consumed kefir was similar to PC, but rats in the kefir group consumed less food and energy than PC ($p = 0.0009$, **Fig. 1B** and $p = 0.0008$ **Fig. 1C**, respectively). Lower feed efficiency was observed in the PC and Kefir group ($p < 0.0001$, **Fig. 1D**).

Regarding the obesity markers, after 10 weeks of the experiment, the animals showed no differences in BMI ($p = 0.215$, **Fig. 1E**). The animals in kefir group had lower thoracic circumference ($p < 0.0001$, **Fig. 1F**). Among animals with induced MetS, the kefir group demonstrated significant reduction in abdominal circumference and delta abdominal circumference when compared to PC ($p < 0.0001$, **Fig. 1G** and $p < 0.0001$, **Fig. 1H,** respectively), presenting similar results to healthy animals.

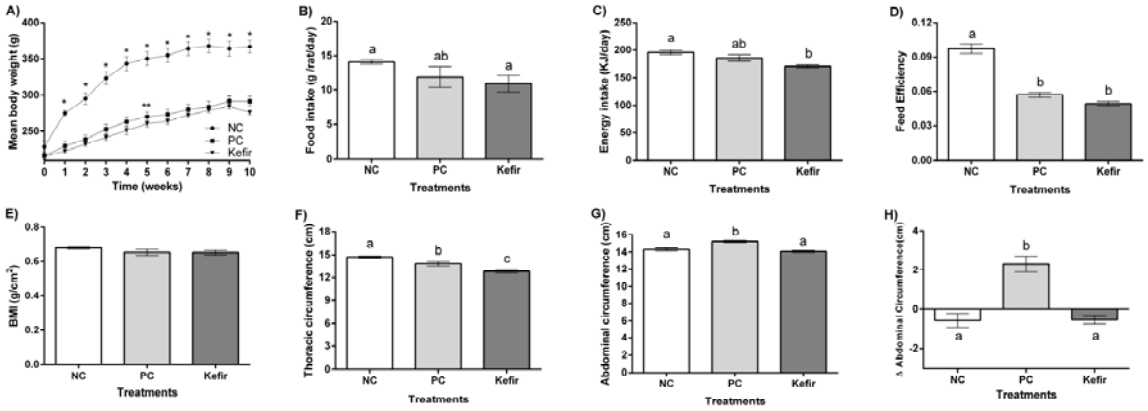


**Fig. 1** – Effect of kefir consumption on the weight change, food intake and biometrics parameters of the SHR animals during the experimental period. Three groups studied include: NC (negative Control); PC (Positive Control) and Kefir group. (A) mean body weight; (B) food intake; (C) energy intake; (D) feed efficiency; (E) BMI, body mass index; (F) thoracic circumference; (G) abdominal circumference; (H) delta abdominal circumference.Values are expressed as means ± SEM (n=10). * $p < 0.0001$, NC significantly different from PC and Kefir group.

*Biochemical analyses*

The results of the clinical analyses are presented in **Table 1**. The consumption of kefir had no effect on serum levels of total cholesterol, HDL-c, LDL-c, VLDL-c, liver total cholesterol, ALT, and creatinine compared to NC and PC ($p > 0.05$).

The consumption of kefir was able to reduce triacylglycerol ($p = 0.035$), liver lipids ($p = 0.008$), liver triacylglycerol ($p = 0.025$), liver weight ($p < 0.0001$) and hepatosomatic index ($p < 0.0001$) when compared to the PC group, which was similar to the NC experimental group (without induction of MetS). Moreover, rats that consumed kefir demonstrated increased levels of AST ($p < 0.0001$), leptin ($p = 0.0007$), calcium ($p = 0.006$) and C reactive protein ($p < 0.0001$). The consumption of kefir was unable to reduce the systolic pressure of the animals and they remained hypertensive throughout the experimental period (**Table 1**).

**TABLE 1**: Effect of kefir supplementation on metabolic parameters measured on the SHR

| Parameters | NC | PC | Kefir | *p* |
|---|---|---|---|---|
| Total cholesterol (mmol/L) | 1.34 ± 0.07 [a] | 1.07 ± 0.04 [b] | 1.15 ±0.04 [ab] | *0.008* |
| HDL cholesterol (mmol/L) | 0.68 ± 0.05 [a] | 0.52 ± 0.01 [b] | 0.55 ± 0.02 [b] | *0.005* |
| LDL cholesterol (mmol/L) | 0.58 ± 0.04 | 0.43 ± 0.03 | 0.49 ± 0.03 | *0.069* |
| VLDL cholesterol (mmol/L) | 0.11 ± 0.005 | 0.10 ± 0.01 | 0.13 ± 0.005 | *0.080* |
| Triacylglycerol (mmol/L) | 0.59 ± 0.02 [a] | 0.70 ± 0.03 [b] | 0.54 ± 0.05 [a] | *0.035* |
| Liver Lipids (mg/g tissue) | 37.41 ± 1.70 [a] | 48.20 ± 3.18 [b] | 39.25 ± 1.94 [a] | *0.008* |
| Liver Total cholesterol (mg/g tissue) | 0.72 ± 0.05 | 0.78 ± 0.08 | 0.72 ± 0.06 | *0.780* |
| Liver Triacylglycerol (mg/g tissue) | 1.60 ± 0.15 [a] | 1.78 ± 0.26 [b] | 1.63 ± 0.25 [a] | *0.025* |
| Liver weight (g) | 12.14 ± 0.31 [a] | 9.97 ± 0.26 [b] | 8.22 ± 0.11 [c] | *<0.0001* |
| Hepatosomatic index | 33.10 ± 0.20 [a] | 34.19 ± 0.26 [b] | 29.80 ± 0.22 [c] | *<0.0001* |
| Leptin (μg/L) | 1.86 ± 0.20 [a] | 1.87 ± 0.24 [a] | 3.80 ± 0.52 [b] | *0.0007* |
| Calcium (mmol/L) | 14.10 ± 1.24 [ab] | 10.86 ± 1.98 [a] | 17.76 ± 2.26 [b] | *0.006* |
| C Reative Protein (mmol/L) | 91.25 ± 1.08 [a] | 57.85 ± 2.88 [b] | 87.22 ± 1.63 [a] | *<0.0001* |
| Systolic Pressure Baseline (mmHg) | 223.18 ± 9.38 | 193.58 ± 10.60 | 204.92 ± 11.85 | *0.148* |
| Systolic Pressure Wk 10 (mmHg) | 215.04 ± 9.77 | 198.61 ± 11.35 | 214.25 ± 6.92 | *0.417* |

Values are expressed as means ± SEM (n=10). [a,b,c] Mean values within a row with unlike superscript letters were significantly different ($p<0.05$; Tukey's post hoc ANOVA statistical analysis).

*Oral Glucose Tolerance Test (OGTT)*

The effects of kefir on incremental changes in plasma glucose at selected intervals (0, 30, 60, 90, and 120 min) after 10 weeks of treatment are shown in **Fig. 2A**. After the animals received the glucose load orally, the treatment groups demonstrated similar outcomes at 30 and 60 minutes ($p > 0.05$). Among animals with induced MetS, the Kefir group showed a significant reduction in incremental glucose at 0 ($p = 0.0003$), 90 ($p = 0.004$) and 120 ($p = 0.0004$) minutes, which were similar to the NC group. The same AUC was noted for the Kefir and NC group. However, Kefir group showed a reduction of approximately 10 % the AUC compared to PC group, which was statistically significant ($p=0.0074$, **Fig. 2B**). The group receiving kefir also showed a decrease in fasting glucose ($p = 0.008$, **Fig. 2C**), fasting insulin ($p = 0.007$, **Fig. 2D**) and HOMA-β ($p = 0.0005$, **Fig. 2F**) when compared to PC group. The differences in HOMA-IR were not significant between the treatments ($p = 0.8070$, **Fig. 2E**).

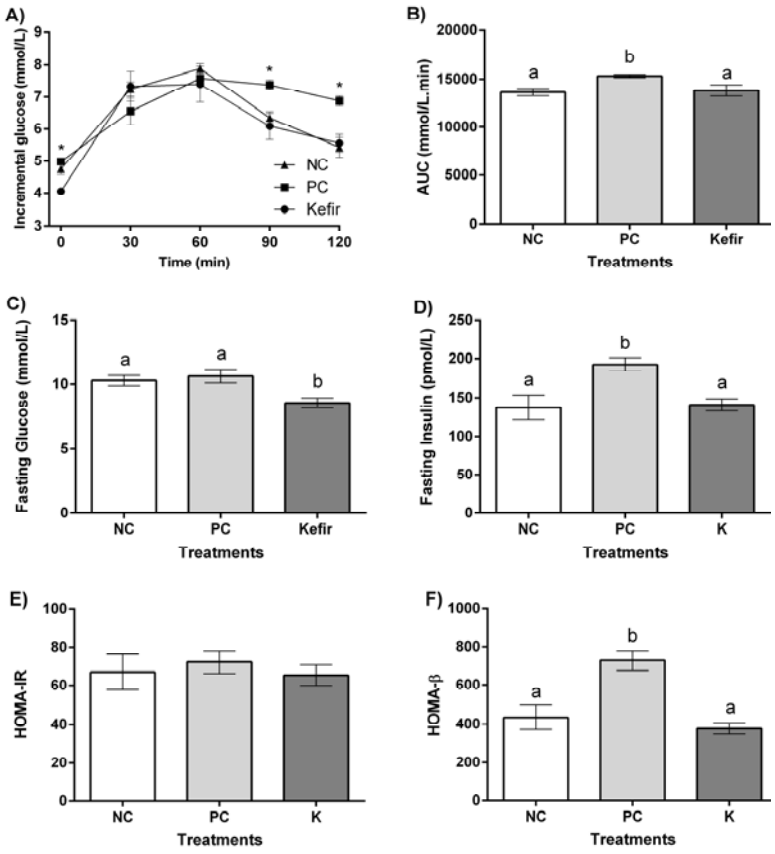


**Fig. 2** – Effect of kefir consumption on tolerance to oral glucose in SHR animals after 10 wk of consumption. (A) The effect of kefir on incremental changes in plasma glucose at selected time intervals 0, 30, 60, 90 and 120 mim. * $p < 0.001$, PC

significantly different from NC and and Kefir groups; (B) The effect of kefir on total area under curves (AUCs) of plasma glucose by oral glucose test tolerance; (C) fasting glucose; (D) fasting insulin; (E) HOMA-IR, homeostatic model assessment-insulin resistance; (F) HOMA-β, beta-cell secretion increase the homeostasis model assessment. Values are expressed as means ± SEM (n=8).

Animals were submitted to OGTT conducted at the beginning and at the end of the experiment. When comparing the baseline (prior to providing the kefir) and experimental values after 10 weeks (**Table 2**), the group receiving kefir had lower blood glucose levels in response to glucose load at 0, 30, 60, 90, and 120 min and a lower AUC ($p < 0.05$). The PC group also showed a decrease in blood glucose levels at 60 and 90 min ($p < 0.05$). The PC group did not show any differences in blood glucose levels at the investigated time points and there was no difference in the AUC ($p > 0.05$).

**TABLE 2**: The effect of kefir supplementation on incremental changes in plasma glucose performed in the beginning and the end of experiment in SHR animals with MetS.

| | | **NC** | **PC** | **Kefir** |
|---|---|---|---|---|
| **T0** | Baseline | 4.94 ± 0.25 | 4.42 ± 0.17 | 5.13 ± 0.16 |
| | Wk 10 | 4.75 ± 0.16 | 4.05 ± 0.09 | 4.35 ± 0.15 |
| | *p* | *0.530* | *0.085* | *0.003* |
| **T30** | Baseline | 8.20 ± 0.89 | 6.70 ± 0.54 | 10.12 ± 0.73 |
| | Wk 10 | 7.25 ± 0.22 | 6.55 ± 0.41 | 7.33 ± 0.46 |
| | *p* | 0.222 | *0.842* | *0.007* |
| **T60** | Baseline | 9.07 ± 0.17 | 7.47 ± 0.33 | 10.77 ± 0.73 |
| | Wk 10 | 7.89 ± 0.14 | 7.64 ± 0.20 | 7.40 ± 0.55 |
| | *p* | *<0.001* | *0.696* | *0.011* |
| **T90** | Baseline | 8.02 ± 0.24 | 6.34 ± 0.38 | 9.45 ± 0.84 |
| | Wk 10 | 7.38 ± 0.15 | 6.30 ± 0.25 | 6.28 ± 0.41 |
| | *p* | *0.014* | *0.880* | *0.026* |

**TABLE 2 (continuation) -**: The effect of kefir supplementation on incremental changes in plasma glucose performed in the beginning and the end of experiment in SHR animals with MetS.

| | | NC | PC | Kefir |
|---|---|---|---|---|
| **T120** | Baseline | 7.52 ± 0.21 | 5.26 ± 0.18 | 6.91 ± 0.37 |
| | Wk 10 | 6.88 ± 0.15 | 5.43 ± 0.31 | 5.56 ± 0.27 |
| | *p* | *0.080* | *0.655* | *0.044* |
| **AUC** | Baseline | 847.73 ± 44.30 | 781.44 ± 23.57 | 1075.31 ± 71.75 |
| | Wk 10 | 850.31 ± 9.09 | 768.81 ± 25.95 | 791.43 ± 37.11 |
| | *p* | *0.947* | *0.635* | *0.019* |

T, time in minutes after the animals received the glucose load orally; Wk, weeks of experiment; AUC, area under curve. Blood glucose levels in animals are expressed in mmol/L. Values are expressed as means ± SEM. $p<0.05$, paired t-test.

*Inflammatory cytokine analyses in adipose tissue*

The analyses of the cytokines in adipose tissue revealed that the animals with MetS receiving kefir had lower levels of the pro-inflammatory cytokine IL-1β compared to PC group (p=0.0053, **Fig. 3A**). On the other hand, the Kefir group showed higher level of pro-inflammatory cytokine IL-6 (p<0.0001; **Fig. 3B**) compared to NC and PC groups. The Kefir group had also higher level of anti-inflammatory cytokine IL-10 (p<0.0307; **Fig. 3C**), compared to PC group.

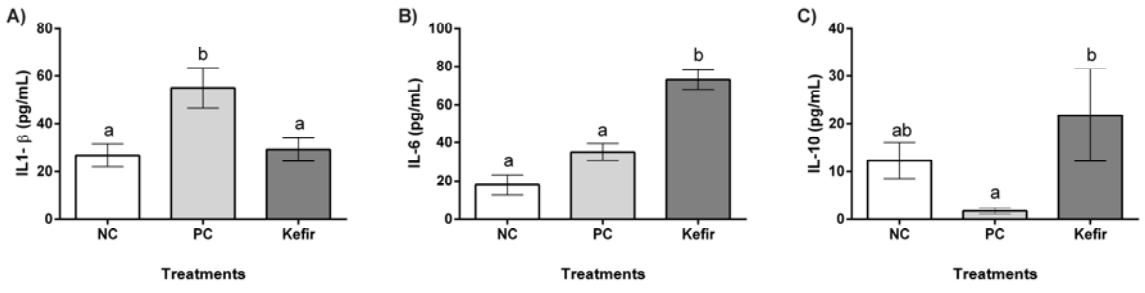


**Fig. 3** – Concentration of cytokines in the intra-abdominal adipose tissue related to the inflammatory process in SHR animals with induction of metabolic syndrome and treated with kefir. **A**- IL1-β cytokine; **B**- IL-6 cytokine; **C**- IL-10 cytokine. Values are expressed as means ± SEM (n=8).

*Oxidant Status Markers in the Liver*

Enzymes involved in the endogenous antioxidant defense system were evaluated to measure the antioxidant activity of kefir (Figure 4). The consumption of kefir was able to decrease the hepatic levels of catalase and superoxide dismutase to the values observed in NC group, when compared to PC group ($p = 0.0002$, **Fig. 4A and** $p < 0.0001$, **Fig. 4B**, respectively). No difference between the three intervention groups was observed in the levels of glutathione S-transferase ($p= 0.3983$, **Fig. 4C**).

The products of lipid and protein oxidation were also evaluated. The animals with induced MetS and supplemented with kefir had decreased levels of malondialdehyde ($p < 0.0001$, **Fig. 4D**) and hydroperoxide ($p = 0.0003$, **Fig. 4E**) when compared to PC group. The consumption of kefir did not alter the levels of protein carbonyl ($p > 0.05$, **Fig. 4F**).

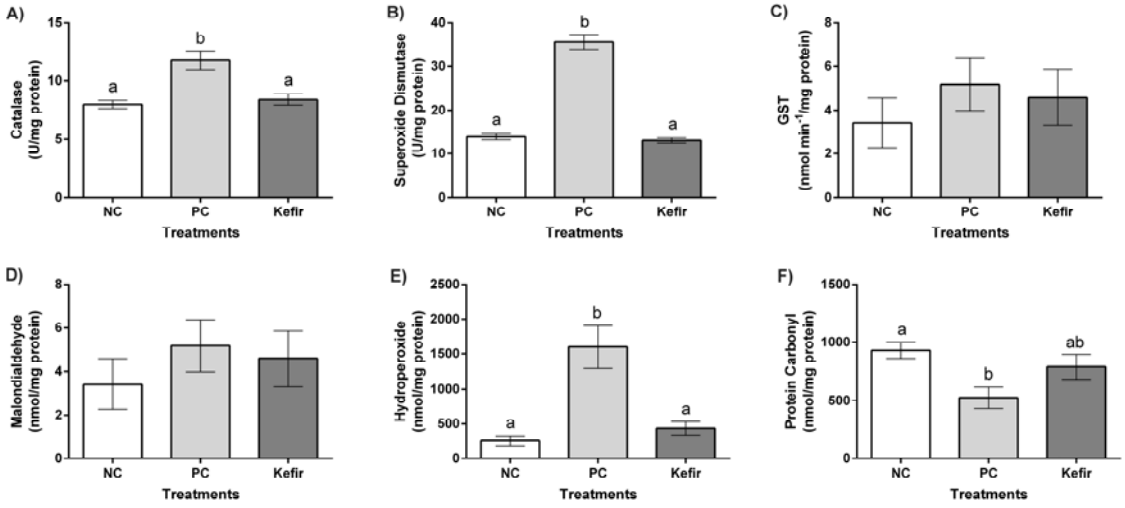


**Fig. 4** – Oxidant status markers in the liver. Effect of treatment with kefir in liver tissue of SHR animals with MetS. (A) catalase activity; (B) superoxide dismutase activity; (C) glutathione S-transferase activity (GST); (D) malondialdehyde; (E): hydroperoxide; (F) protein Carbonyls. The data are expressed as means ± SEM (n=8). $^{a,b,c}$ Unlike superscripts in columns indicate statistical difference between the groups ($p < 0.05$); common superscripts indicate no statistical difference; ANOVA followed by Tukey's test.

*Histological analyses*

The results of the histological analyses of pancreatic and adipose tissues are presented in **Fig. 5**. The induction of metabolic syndrome with MSG was able to influence lipid deposition in adipose tissue and area of islets in pancreatic tissue. Animals with MetS

had lower area of islets ($p < 0.0001$, **Fig. 5D**) and greater adipocyte area ($p < 0.0024$, **Fig. 5H**) compared to healthy animals (NC group). The consumption of kefir did not influence the histological analysis of adipose and pancreatic tissue

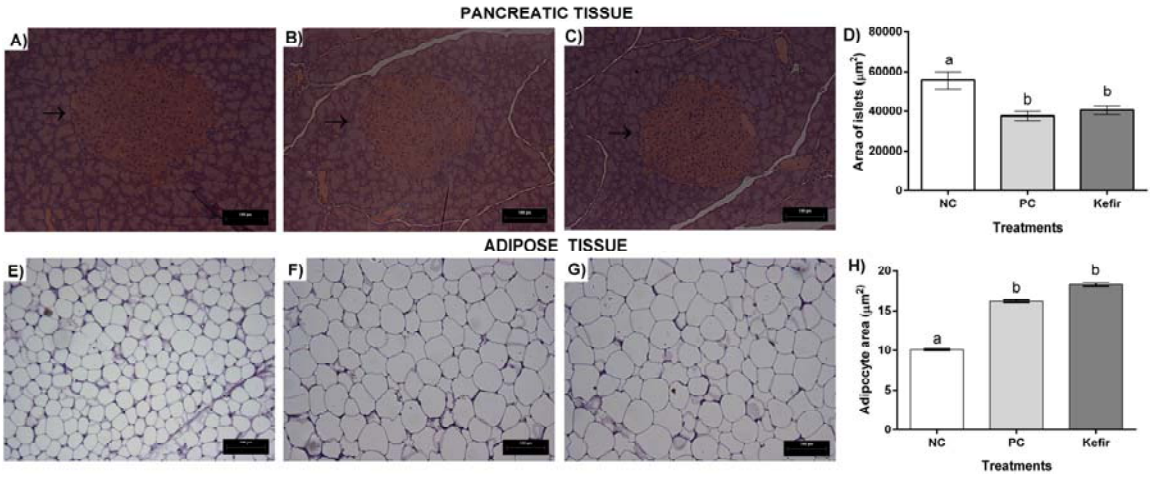


**Fig. 5** – Representative photomicrographs of histological section of the intra-abdominal adipose and pancreatic tissue of SHR animals with MetS and treated with kefir, and stained with hematoxylin/eosin and observed under light microscope. The pancreatic tissue of SHR animals: (A) NC, negative control; (B) PC, positive control; (C) kefir group; (D) area of pancreatic islets. The adipose tissue of SHR animals: (E) NC, negative control; (F) PC, positive control; (G) kefir group; (H) adipocyte area. Values are the means ± SEM (n = 6). Bar =50µm.

### 6.5. Discussion

During recent years an increased incidence of chronic diseases and occurrence of MetS created a need to find alternatives to conventional therapies, which could be easily incorporated into the diet and at a low cost. Kefir represents a potential food-based tool for the prevention and treatment of such pathologies. Importantly, our study is the first that assesses the effect of kefir administration on the MetS parameters. In particular, we assessed biochemical, obesity, blood pressure, OGTT, inflammatory cytokines, antioxidant system, peroxidation biomarkers and histological analyses in an animal model of MetS with and without supplementation of kefir.

In our study, the animals that received MSG had lower body weight gain. The consumption of kefir did not influence body weight in MetS animals. On the other hand, the healthy rats demonstrated increased body weight. Although the three animal study

groups did not differ in BMI, the consumption of kefir resulted in decreased abdominal circumference, thoracic circumference and delta abdominal circumference in animals that received MSG, indicating that kefir is able to influence the body fat distribution of rodents. According to Després and colleagues [39] the intra-abdominal adiposity drives the progression of multiple cardiometabolic risk factors independently of body mass index and the prognostic importance of high waist circumference has been recognized within the diagnostic criteria to identify individuals with features of the MetS. The reduction in abdominal circumference observed in animals supplemented with kefir is an important protective mechanism. Additionally, the histological analysis revealed that the neonatal administration of MSG increased area of adipocytes in intra-abdominal adipose tissue but the consumption of kefir was unable to reverse this process. According to Ho and coworkers [40], kefir inhibits adipocyte differentiation through suppression of transcriptional factors, adipogenesis related genes, as well as enzymes and could be considered as a potential candidate for the regulation of metabolic syndrome, situation not found in our study.

The MetS is a group of clinical risk factors comprising atherogenic dyslipidemia (low HDL-c and high triglycerides levels), elevated blood pressure, elevated plasma glucose, a prothrombotic state, and a proinflammatory state accompanied by insulin resistance and oxidative stress that may play an important role in the pathogenesis of MetS [41; 42]. Functional foods, with particular probiotic kefir have a potential in controlling these metabolic dysfunctions. In our study, animals that received kefir with MetS showed a significant reduction in plasma levels of triacylglycerol. In agreement with our results, Huang and coworkers (2013) [43] investigated the effect of *Lactobacillus plantarum* Lp27 isolated from Tibetan kefir grains on hypercholesterolemic rats for 4 weeks. The animals showed decrease in triacylglycerol, total cholesterol and LDL-c in serum, triacylglycerol and total cholesterol in liver. In another study, three lactobacilli strains isolated from kefir grains (*L. acidophilus LA15, L. plantarum B23, L. kefiri D17*) were able to reduce triacylglycerol, total cholesterol and LDL-c in serum and increase the fecal excretion of triacylglycerol and total cholesterol [44]. The consumption of kefir in our study animals with induced MetS also reduced the total lipids and triacylglycerol in liver, demonstrating that kefir is an important protective agent. Several lines of evidence indicate that hepatic triacylglycerol accumulation is also a causative factor involved in hepatic insulin resistance. In addition, it involves complex interactions between endocrine, metabolic and

transcriptional pathways that are involved in triacylglycerol induced hepatic insulin resistance, and passively and actively in the metabolic derangements of the metabolic syndrome [45].

A significant decrease in the incremental AUCs of glucose concentration after glucose loading during OGTT were seen in rats fed with kefir, indicating that the ability of insulin to stimulate glucose disposal is markedly impaired in peripheral tissues associated with insulin resistance by MSG. The ability to maintain normoglycemia primarily depends on two factors: the capacity of pancreatic β-cells to secrete insulin (β-cell function) and the sensitivity of glucose-utilizing tissues to the prevailing insulin concentration (insulin sensitivity) [46]. Although the animals showed no statistical difference in HOMA-IR, we demonstrate that animals with MetS treated with kefir showed lower activity of β-cells in the pancreas (lower HOMA-β) and hence a 20 % reduction in fasting insulin and 25 % reduction in fasting insulin, compared to animals receiving saline, which was similar to the situation observed in healthy animals (NC group).

In addition, higher plasma levels of calcium were observed in animals that consumed kefir. Microbial fermentation may increase the bioavailability of different nutrients due to the proteolysis in milk and the degradation of proteins, which enlarges the area susceptible to enzymes and also slows down the gastrointestinal transit time [47]. A calcium-rich diet is known to improve insulin sensitivity [48; 49]. Furthermore, observational studies have shown inverse associations between dairy intake and the prevalence of insulin resistance syndrome and type 2 diabetes mellitus. According to Tremblay and collaborators [50] adequate calcium and dairy food intake is part of a series of good daily life practices which contribute to optimal health and also reduce the necessity to rely on hyperinsulinemia to maintain homeostasis. According to Hadisaputro and coworkers the probiotic kefir protects against glucotoxicity and lipotoxicity and reduces the occurrence of hyperglycemia associated with the synergism of the bioactive compounds found in the fermented drink [14]. [51] Kefir can also stimulate glucose uptake in L6 skeletal muscle cells *in vitro* and reduce the reactive oxygen species that participate in insulin resistance. In addition, kefir activates PI3-kinase (a key enzyme in dysfunctional insulin signal transduction) or other upstream molecules in the insulin signaling pathway, which results in the augmentation of glucose uptake. The anti-oxidative effect of kefir may have been one of the factors that enhanced glucose uptake in L6 myotubes [51]. Kefir contains potential antioxidants especially peptides derived from

milk proteins that interact with a wide range of species directly responsible for oxidative damage, however, the mechanism of action is not clearly understood [52].

Oxidative stress is associated with a number of components of MetS [53]. Therefore, the use of antioxidant foods is an important alternative for the management of MetS. According Liu and co-workers [54], kefir possesses antioxidant activity and is a potential candidate as a useful natural antioxidant supplement in the human diet. To evaluate the antioxidant potential of kefir in rats with MetS, we evaluated the enzymes involved in the endogenous antioxidant system and the protein and lipid oxidation products. In our study, the antioxidant activity of kefir was verified by decreased levels of products of lipid oxidation hydroperoxide and malondialdeyde and consequently lower demand for antioxidant enzymes: superoxide dismutase and catalase. Kefir was effective in reducing oxidative stress in liver. A group lead by Guven compared the antioxidative consequence of kefir and vitamin E consumption against oxidative damage of $CCL_4$ in an animal model. The authors observed a higher decrease in MDA levels in liver after supplementation with kefir compared to vitamin E [55]. Another study demonstrated that consumption of kefir markedly prevented oxidative-induced cell death in cultured cells derived from mouse brain [56]. In accordance with our findings, Punaro and coworkers [57] observed that consumption of kefir for two weeks in diabetic rats resulted in reduced lipoperoxidation and increased oxide nitric bioavailability, suggesting that this fermented milk can attenuate the deleterious effects of diabetes through the control of oxidative stress. Thus, the antioxidant activity of kefir may be responsible for a significant portion of the health-promoting effects attributed to this probiotic.

In MetS, numerous inflammatory markers are highly associated with the degree of obesity and insulin resistance [58; 59]. Many of the inflammatory markers found in the plasma of obese individuals appear to originate from adipose tissue. It therefore suggests that obesity is a state of chronic low-grade inflammation initiated by morphological changes in adipose tissue [15]. Adipose tissue secretes several hormones such as leptin and adiponectin and a variety of adipocytokines leading to the development and/or aggravation of insulin resistance [60; 61; 62] and the development of MetS [63]. In our study the neonatal administration of MSG in SHR rats was able to influence the concentration of inflammatory cytokines in adipose tissue. The daily consumption of probiotic kefir for 10 weeks reduced the IL-1β levels in adipose tissue which reflected situation in healthy animal. IL-1β is one of the major proinflammatory cytokine that is produced by monocytes

and macrophages [64] and several studies have shown that IL-1β deteriorates peripheral insulin sensitivity and inhibits insulin production by the pancreas. IL-1β concentration is elevated in diabetic individuals, which correlates with MetS [65]. Finally, the expression of both IL-1β and its receptor are increased in the visceral adipose tissue of obese subjects [66], corroborating our findings that the animals which received kefir had lower levels of IL-1β and also had lower abdominal circumference compared to PC group. Ours study demonstrates that the level of pro-inflammatory cytokine IL-6 was increased in adipose tissue of animals which consumed kefir. The endocrine cytokine IL-6 is a mediator of proinflammatory signaling from adipose tissue in obesity, as noted earlier. On the other hand, the consumption of kefir was able to increase twelve times greater the levels of IL-10 in adipose tissue, which is an important anti-inflammatory cytokine. The observed high level of IL-6 might have resulted from the markedly increased IL-10 or vice versa, which may suggest homeostatic effect on the immune system. It has been reported that probiotic effect on immunity is strain specific [67]. In addition, probiotic intervention depends on the gut microbial composition of the host [68], which may then specifically affect the immune system. These observations call for furter studies such as evaluation of other anti-inflammatory markers and gut microbiota, which may have contributed to the effect reported in the present study. Hadisaputro et al.[14] evaluated the effect of kefir on the immune responses of Wistar rats with hyperglycemia induced by streptozotocin. The group found that kefir supplementation for 30 days affected blood glucose levels and influenced proinflammatory cytokines, which was in agreement with our results.

The antihypertensive effects of probiotics have been observed in clinical and animal studies [69]. Unlike previous studies, the consumption of kefir in the present study was unable to reduce the blood pressure of the animals and high pressure was maintained during the entire experimental period. The choice of an experimental model of obesity associated with neuroendocrine disruptions (induced via the application of neonatal MSG) and hypertension in SHR mimics, in part, the conditions of MetS. These animals have high blood pressure, are more resistant to peripheral insulin, and have increased central adiposity, as it has been demonstrated in previous studies [19; 20; 22; 70].

In the present study, the animals received 1 mL of kefir by gavage. Making an extrapolation to the human diet and considering the adult man of approximately 70 kg body weight, the volume of this probiotic drink for body weight represents a daily dose of 230

mL of kefir, a quantity easily incorporated in a diet. Using an MRS medium which favors the growth of most lactic acid bacteria (LAB), we found that this group was present in kefir at a level of 2.7 x $10^7$ CFU/mL. Such number of colony forming units is desirable for the physiological effects of this probiotic product. If consumed daily, cultured products sold with any health claim benefits should meet the criteria of the suggested minimum number of $10^6$ CFU/g at the time of consumption [71].

Some researchers believe that the various health-promoting properties of kefir outweigh those of other fermented products. The important active constituents of kefir include beneficial microorganisms (bacteria and yeasts) that belong to strains which species have been characterized as probiotics, and bioactive components often derived from the microbial activity. Thus, the functional role of kefir may be either direct, through an interaction with the consumed microorganisms (probiotic effect), or indirect, as a result of the action of microbial metabolites like vitamins, proteins, bioactive peptides, oligosaccharides and organic acids, generated during the fermentation process (biogenic effect). The consumption of kefir is capable of modulating the gut microbiota of the host [72; 73]. The gut microbiota also regulates many aspects of innate and acquired immunity, protecting the host from pathogen invasion and chronic inflammation. Investigators have associated imbalances in the gut microbiota with susceptibility to infections, immune-based disorders and recently and more importantly, with obesity and insulin resistance, providing strong scientific evidence for using probiotics in the formulation of dietary strategies for the management of MetS [17].

## 6.6. Conclusions

Taken together, we conclude that kefir supplementation in MetS in SHR for 10 weeks was able to reduce thoracic and abdominal circumference of animals, as well as plasma triglycerides, liver lipids and liver triglycerides. Moreover, animals that consumed kefir had lower fasting glucose, fasting insulin levels, reduced insulin resistance (OGTT and AUC), reduced inflammatory cytokine expression in adipose tissue (IL-1β), increased anti-inflammatory cytokine (IL-10) and decreased oxidation products such as malondialdehyde and hydroperoxides. In this context, the kefir supplementation in animals with MetS showed anti-inflammatory action, decreased insulin resistance and oxidative stress, important components of MetS. Thus, kefir represents a promising potential in the management of MetS, as well as being low-cost and easy accessible probiotic product. Our

results clearly demonstrate that fermented probiotic products may be beneficial in the management of metabolic disorders. Further studies are necessary to explore the role of kefir on gut microbiota modulation and mucosal health among others, before clinical applications of kefir in MetS patients.

**Competing interests**

The authors declare no conflict of interest.

**Authors' contributions**

The authors' contributions are as follows: D.D.R. designed the experiments, performed data collection and analysis, interpreted the data and wrote the manuscript. Ł.M.G. contributed to interpretation of data and critical revision; S.M.F., A.C.M.F., M.M.D., N.P.S. and L.L.S., designed the experiments, and performed data collection and analysis; C.A.N., performed and interpreted histological analyses and reviewed the paper; L.L.O., performed and interpreted inflammatory cytokine analyses in adipose tissue and reviewed the paper; C.L.L.F.F., contributed to critical revision and to approving the final version; M.C.G.P., contributed to conception and design of the study, interpretation of data, critical revision and to approving the final version.

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# 7. ARTICLE 3: Kefir reduces endotoxemia, intestinal permeability and increase fecal short-chain fatty acids of rats with induced metabolic syndrome

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**Running title**: Kefir reduces endotoxemia and **intestinal permeability**

**Abbreviations**: Metabolic syndrome (MetS); monosodium glutamate (MSG); colony forming unit (CFU); Lactic acid bacteria (LAB); body mass index (BMI); high-lipoprotein cholesterol (HDL-C); colony forming unit (CFU); short-chain fatty acids (SCFA); angiotensin-converting enzyme (ACE)

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## 7.1. Abstract

Kefir is a probiotic drink obtained by fermenting milk with kefir grains. It consists of bacteria and yeasts that coexist in a complex symbiotic association. This study aimed to assess the effect of kefir on metabolic parameters, intestinal permeability, short-chain fatty acids in feces and histological and morphometric analysis in the bowel. Twenty male SHR received neonatal intradermal injections of MSG and were divided into two groups. For the negative control, ten male SHR received neonatal intradermal injections of saline solution (0.9 % NaCl solution). The rats were randomly divided into three groups (10 animals in each group): negative control (NC, 1mL saline solution/day), positive control (PC, 1mL saline solution/day) and kefir group (1 mL kefir/day). Feeding was carried out by gavage and lasted 10 weeks. Body weight, food intake, metabolic parameters, intestinal permeability, short-chain fatty acids in feces and histological and morphometric analysis in the bowel. The kefir supplementation in an animal model of MetS reduced triglyceride levels and intestinal permeability. Higher levels of total short-chain fatty acids, acetic and butyric acids was found in the kefir group. The reduced levels of plasma lipopolysaccharide (LPS) were detected in animals receiving kefir. The probiotic kefir was able to improve the intestinal mucosa in the small intestine of animals and not found differences in the large intestine. Kefir represents a promising potential in the management of MetS, at the same time being a low-cost probiotic that is easily incorporated into the diet.

**Keywords***: kefir, probiotic, metabolic syndrome, lipopolysaccharide, intestinal permeability, LPS, short-chain fatty acids, bowel histology,*

## 7.2. Introduction

Kefir is a probiotic drink obtained by fermenting milk with kefir grains. It consist of bacteria and yeasts that coexist in a complex symbiotic relationship (Franco et al., 2013). Some researchers believe that the various health-promoting properties of kefir outweigh those of other fermented products. Studies concerning kefir have attracted the public because of kefir's potential on health, low cost and the easy preparation. The active constituents of kefir include bacteria, yeasts that belong to strains of species that have been characterized as probiotics, and bioactive components often derived from the microbial activity. Thus, the functional role of kefir may be either direct, through interactions with the consumed microorganisms, or indirect, as a result of the action of microbial metabolites such as vitamins, proteins, peptides, oligosaccharides, and organic acids, generated during the fermentation process.

The gut microbiota regulates many aspects of innate and acquired immunity, protecting the host from pathogen invasion and chronic inflammation (Guzel-Seydim, Kok-Tas, Greene, & Seydim, 2011). The consumption of kefir is capable of modulating the gut microbiota of the host (Yaman, Ulukanli, Elmali, & Unal, 2006). The properties observed in kefir include: antimicrobial and antioxidant activity, hypotensive action, anti-carcinogenic and anti-mutagenic properties, improved gastrointestinal function, cholesterol lowering effects and immunoregulatory effects (Guzel-Seydim et al., 2011). Recently, investigators have connected imbalances in the gut microbiota with susceptibility to infections, immune-based disorders and, more importantly, with obesity and insulin resistance, providing strong scientific evidence for the use of probiotics and prebiotics in the formulation of dietary strategies for the management of MetS (Mallappa et al., 2012).

MetS is a complex of interrelated risk factors for cardiovascular disease and diabetes, such as hyperglycemia, hypertension, high triacylglycerol levels, low HDL-cholesterol (HDL-c) levels, and abdominal obesity (WHO, 2002). Evidences show that probiotic bacteria intake could be a useful tool in the control of MetS (Bogsan et al., 2011).

Intestinal barrier function has been viewed as an interface between health and disease (Groschwitz & Hogan, 2009). There is an association between altered intestinal permeability, adiposity and insulin resistance. Therapies aimed at reduction of intestinal permeability may be crucial in the context of obesity and MetS (Teixeira et al., 2012). The disrupted intestinal barrier can lead to an increased permeability, allowing the entrance of lipopolysaccharides (LPS), among others, into the body. They can cause the release of pro-

inflammatory cytokines in the body, leading to systemic inflammatory diseases, like MetS (Hemert, Verwer, & Schütz, 2013). Recently, there has been great interest in the use of food supplements containing probiotics to combat specific disorders. This led us to the question on the role of kefir in the control and management of MetS.

The use of SHR associated with MSG is an important animal model for studying MetS (Aleixandre de Artinano & Miguel Castro, 2009). In the present study, we evaluated the effect of kefir supplementation in MSG-induced MetS in SHR. In particular, our aim was to determine the protective effect of kefir on metabolic parameters, intestinal permeability, short-chain fatty acids in feces and histological and morphometric analysis in the bowel.

## 7.3. Material and methods

*Kefir preparation*

Kefir particles (grains of kefir, obtained from a private household in Viçosa, Brazil) were washed with distilled water, inoculated in integral cow's milk, pasteurized type C (nutritional composition: 3.5% protein, 5% carbohydrate, 3% fat, 1.2 mg/mL calcium, 0.6 mg/mL sodium, giving total of 2.56 kJ/mL) and stored at 4°C. The kefir beverage was prepared by adding kefir grains to fresh milk at 5% (wt/wt) under incubation at 25-28°C for 24 hours. The kefir grains were separated from the fermented milk, and were filtered and washed before reuse for subsequent fermentations (Urdaneta et al., 2007). The fresh kefir beverage offered to the animals presented the following physicochemical composition: pH 4.10 ± 0.10; high acidity, 0.461 ± 0.06 g/100 g of lactic acid; lipids 3.30 ± 0.16 g/100 g, proteins 3.00 ± 0.01 g/100 g. The lactic acid bacteria (LAB) were present in kefir at the levels of 2.78 x $10^7$ CFU/mL and yeasts at 2.94 x $10^8$ cell/mL, as determined by selective agar plating. Each rat orally administered with 1 mL of kefir, received 1.36 x $10^8$ CFU/Kg body weight/day for 10 weeks.

### Animals and Experimental Design

*Induction of MetS*

Twenty SHR male newborns were obtained from the Central Animal House at the Center of Biological Sciences at the Federal University of Viçosa. For the induction of MetS, two-days-old male rats received intradermal injections of monosodium glutamate (MSG, 4 mg/g body weight, Sigma Co., St. Louis, MO), until they completed the age of six

days, which gave a total of five applications, according to Cunha et al. (2010). For negative control, 10 rat's two-days-old male rats received intradermal injections of saline solutions (0.9 % NaCl/day) in same conditions. After weaning, the animals were housed in collective cages for 100 days with food and water *add libitum* until the MetS was developed.

*Animals, housing and feeding*

When the animals were three months old, they were randomly divided into three groups (10 animals in each group): Negative Control (NC, 1mL 0.9 % sodium chloride solution/day), Positive Control (PC, 1mL 0.9 % sodium chloride solution/day) and Kefir group (1 mL kefir/day); feeding was carried out by gavage. Each rat received 1 mL of kefir orally which corresponded to 1.36 x $10^8$ CFU/Kg body weight/day of LAB, for the period of 10 weeks. Making an extrapolation to the human diet and considering that the adult man have a body weight of approximately 70 kg, the volume of the probiotic drink for body weight represents a normal daily dose of 230 mL of kefir, a quantity that is easily incorporated in a diet.

The animals were housed in individual cages and maintained under standard conditions (12h light/12h dark cycle, 22°C ± 1 room temperature). Standard Nuvilab® food (composition: 19.0% protein, 56.0% carbohydrate, 3.5% fat, 4.5% cellulose, 5.0% vitamins and minerals, giving a total of 13.87 kJ/g) and filtered water were provided *ad libitum*. Food consumption was measured daily and body weights were evaluated each week during the entire experimental period. The rats were anesthetized with Halothane (Tanohalo®, Cristália) after 10 weeks of kefir administration. The tissues were removed for histological analyses and stored at -80°C for further analysis.

*Biochemical analyses*

Blood samples were centrifuged at 700 x g for 10 minutes to obtain serum and heparin and the tubes were stored at -80°C for subsequent analysis. Total cholesterol, high-density lipoprotein cholesterol (HDL-c), triacylglycerol, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and glucose were analyzed in serum using diagnostic test kits (Bioclin®, Diagnostics®, Belo Horizonte, Brazil) and autoanalyzer equipment (COBAS MIRA Plus, Roche Diagnostic Systems, Branchburg, NJ).

*Blood pressure — tail-cuff method*

To calm down the animals and dilate the tail blood vessels before measurements, the rats were placed inside a warm chamber (about 40 °C) for 5 min. Measurements of arterial blood pressure (systolic) were carried out at least five times for each animal and four nearest was considered, to give better reliability of the data. The blood pressure was performed at the beginning and at the end of the experiment. Resting systolic blood pressure was measured in conscious rats using a tail-cuff pressure meter (LE5001, Panlab, Harvard Apparatus, Wood Dale, IL, USA).

*The intestinal permeability test*

The permeability of the intestine to sugars was investigated using the lactulose-mannitol test and were performed before and after week 10 of the experimental period. The animals were placed in individual metabolic cages without water and diet for the period of four hours. Thereafter, rats received by gavage, 200 mg lactulose and 100 mg mannitol dissolved in 1 ml of water. During the following eight hours, the animals were hydrated by gavage every two hours with 2 mL of water to control hydration, and the urine was collected and quantified to estimate intestinal permeability; 2.5 mL of urine was placed to a small flask with thimerosal to prevent bacterial growth and the solution was stored at -20°C until further analysis. Urinary excretion rates of lactulose and mannitol were analyzed using the high performance liquid chromatography (HPLC) System (Shimadzu LC-10AD Liquid Chromatography) with a Shimadzu SPD-6A UV-VIS detector (Shimadzu, Kyoto, Japan). The results are reported in percentage of urinary excretion of both probes. The final result of the test is the ratio of administered test probes, since this is a more accurate indicator of permeation.

*Lipopolysaccharide determination*

The Limulus Amebocyte Lysate (LAL) commercial kit (Hycult Biotech, The Netherlands) was used to quantify plasma LPS concentration. Plasma samples were heated (75°C) for 5 min. Fifty microliters of undiluted plasma and prepared standards were pipetted into the pyrogen-free microplate. Absorbance was read at 405 nm (Multiskan Go, Thermo Scientific, USA). Reagents were added according to the manufacturer´s instructions. Absorbance was read again. A standard curve and its equation (R2>0.97) were

generated by plotting the concentration of standards (log10) and their absorbance. Plasma LPS concentrations (endotoxins units per milliliter, EU/mL) were estimated using the delta of absorbance (Δ=final absorbance - initial absorbance).

*Quantification of short-chain fatty acids (SCFA) by high-performance liquid chromatography (HPLC), and fecal pH*

The feces of all the animals were collected from the cecum at the time of euthanasia and were stored at 20ºC for further analysis of SCFA (acetate, propionate and butyrate). The material was extracted and analyzed according to the method described by Smirick-Tjardes et al. (2003). For each animal, an aliquot of feces was diluted in distilled water (100 mg/mL), homogenized, and the pH was measured with a portable digital pH meter (Instrutherm, PH-1900, Brazil) at a room temperature (Bedani et al., 2011).

*Histological analyses*

Immediately after euthanasia, ileum and proximal colon samples were excised and rinsed with ice-cold physiological saline for histological evaluation. Tissue samples of approximately 0.5 cm in length were excised from the ileum (2 cm proximal to the caecum), and colon (2 cm distal to the caecum), and fixed in Carson's formalin (Carson, Martin, & Lynn, 1973). After dehydration in an increasing gradient of ethanol, the ileum was embedded in hydroxyethyl methacrylate resin (Historesin®, Leica) and stained with stained in a blue solution of toluidine/sodium borate 1%. The colon tissue was dehydrated in an increasing gradient of ethanol, diaphanized in xylol and embedded in paraffin and stained with Alcian Blue-PAS. In both tissues, twenty images per animal (120 images in each group) were captured. Morphometric analysis to determine the height and width of villous and crypt depth in ileum tissue were performed according to Rosa et al. (2010). In proximal colon samples, crypt depth and mucosa thickness were measured according to Kabeir et al. (2008).

*Ethics*

The study was approved by the Ethics Committee of the Department of Veterinary Medicine of the Federal University of Viçosa (number process: 107/2011) and the

experiments were performed according to the Ethical Principles in Animal Experimentation, adopted from the National Council for the Control of Animal Experimentation.

*Statistical analyses*

All statistical calculations were performed using GraphPad Prism, Prism 5 for Windows, version 6.01 (Graph-Pad Software, Inc., San Diego, CA, USA). Variables were compared between groups using One-way analyses of variance (ANOVA) followed by Tukey's test or using the Kruskal-Wallis test followed by Dunn's test, as appropriate. Paired t-tests were performed to compare intestinal permeability in baseline and week 10. Results are presented as mean values with standard errors and were considered statistically significant at $p < 0.05$.

## 7.4. Results

*Food intake and biochemical analyses*

Food intake and biochemical analyses of the different treatments are presented in **Table 1**. After 10 weeks of treatment, lower body weight ($p < 0.0001$) and gain weight ($p < 0.0001$) were observed in animal's induced metabolic syndrome with MSG. Rats from the kefir group consumed less food than rats from the milk group ($p=0.013$). Rats from the kefir group consumed less food than rats from the NC group ($p=0.0009$). The consumption of kefir had no effect on serum levels of total cholesterol, HDL-c, ALT, and creatinine compared to PC ($p > 0.05$). However, the consumption of kefir was able to reduce triacylglycerol ($p = 0.035$) and fasting glucose ($p = 0.008$) compared to PC group, animals with MetS. Regarding the assessment of the animals' blood pressure, the consumption of kefir was unable to reduce the systolic pressure of the animals compared to unfermented to NC and PC, and the animals remained hypertensive throughout the experimental period (**Table 1**).

**Table 1**: Effect of kefir supplementation on metabolic parameters measured on the SHR

| **Parameters** | **NC** | **PC** | **Kefir** | ***p*** |
|---|---|---|---|---|
| Initial body weight (g) | 229.10 ± 7.32 | 216.80 ± 4.58 | 215.77 ± 4.01 | *0.139* |
| Final body weight (g) | 366.90 ± 8.89 [a] | 291.62 ± 7.02 [b] | 275.88 ± 3.19 [b] | *< 0.0001* |
| Gain of weight (g) | 137.80 ± 1.57 [a] | 74.82 ± 2.44 [b] | 60.11 ± 0.82 [b] | *< 0.0001* |
| Food intake (g/rat/day) | 14.11 ± 0.30 [a] | 11.90 ± 1.53 [ab] | 10.93 ± 1.22 [b] | *0.0009* |
| Total cholesterol (mmol/L) | 1.34 ± 0.07 [a] | 1.07 ± 0.04 [b] | 1.15 ±0.04 [ab] | *0.008* |
| HDL cholesterol (mmol/L) | 0.68 ± 0.05 [a] | 0.52 ± 0.01 [b] | 0.55 ± 0.02 [b] | *0.005* |
| Triacylglycerol (mmol/L) | 0.59 ± 0.02 [a] | 0.70 ± 0.03 [b] | 0.54 ± 0.05 [a] | *0.035* |
| Fasting Glucose (mmol/L) | 10.28 ± 0.40 [a] | 10.63 ± 0.52 [a] | 8.61 ± 0.37 [b] | *0.008* |
| AST ( μKat/L) | 2.37 ± 0.14 [a] | 2.17 ± 0.12 [a] | 3.45 ± 0.15 [b] | *<0.0001* |
| ALT (μKat/L) | 1.16 ± 0.09 | 0.96 ± 0.04 | 1.01 ± 0.07 | *0.205* |
| Creatinine (μmol/L) | 35.58 ± 2.02 | 27.78 ± 1.01 | 34.86 ± 2.65 | *0.055* |
| Systolic Pressure Baseline (mmHg) | 223.18 ± 9.38 | 193.58 ± 10.60 | 204.92 ± 11.85 | *0.148* |
| Systolic Pressure Wk 10 (mmHg) | 215.04 ± 9.77 | 198.61 ± 11.35 | 214.25 ± 6.92 | *0.417* |

AST, aspartate aminotransferase; ALT, alanine aminotransferase. Values are expressed as means ± SEM (n=10). [a,b,c] Mean values within a row with unlike superscript letters were significantly different ($p<0.05$; Tukey's post hoc ANOVA statistical analysis).

*The intestinal permeability test and LPS*

The data obtained from the small-intestine permeability test after and before 10 weeks of supplementation with probiotic kefir are shown in **Table 2**. At the time baseline prior to supplementation with kefir by gavage, the animals already had higher levels of excretion of lactulose ($p = 0.001$) and L / M ratio ($p < 0.0001$). After 10 weeks of supplementation with kefir, animals had higher excretion of mannitol ($p = 0.003$), but lower levels of L / M ratio was found in the kefir, compared to NC and PC groups ($p= 0.001$). When comparing the levels of excretion of sugars before and after the experimental period, the NC and NP groups showed an increase in L / M ratio ($p < 0.0001$ and $p = 0.0005$, respectively), and was not found differences in group kefir.

Additionally, the consumption of kefir was able to reduce the plasma levels of LPS compared to the PC group ($p = 0.003$, **Figure 1**), similar to healthy animals (NC group).

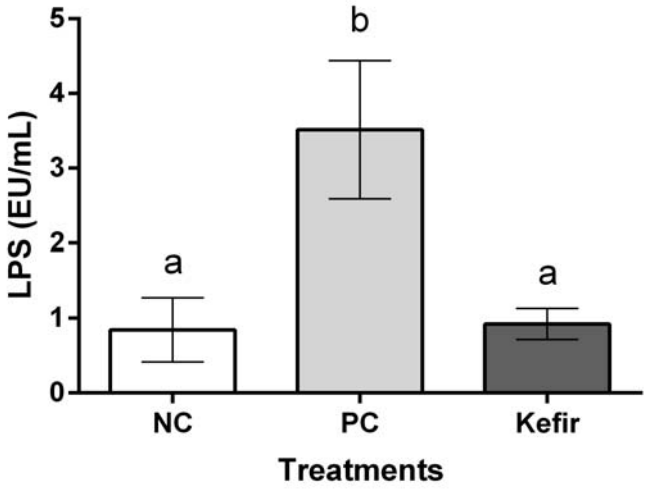


**Figure 1** – Effect of 10-week consumption of kefir on Lipopolysaccharides levels (LPS). Values are expressed as means ± SEM (n=8). [a,b,c] Differences among treatments are indicated by different letters and were considered statistically significant at $p<0.05$; Tukey's post hoc ANOVA was used.

**Table 2**. Intestinal permeability parameters in SHR with induced MetS.

| **Parameter** | **NC** | **PC** | **Kefir** | *p** |
|---|---|---|---|---|
| Lactulose excretion baseline (%) | 0.52 ± 0.04 [a] | 1.12 ± 0.20 [a] | 1.56 ± 0.21 [b] | *0.001* |
| Lactulose excretion Wk 10 (%) | *1.39* ± 0.09 | *1.58* ± 0.42 | *1.36* ± 0.11 | *0.817* |
| *p#* | *< 0.0001* | *0.353* | *0.500* | |
| Mannitol excretion baseline (%) | 3.24 ± 0.44 | 2.68 ± 0.51 | 2.02 ± 0.26 | *0.152* |
| Mannitol excretion Wk 10 (%) | 1.0 ± 0.05 [ab] | *0.82* ± 0.07 [a] | *1.35* ± 0.14 [b] | *0.003* |
| *p#* | *0.001* | *0.011* | *0.185* | |
| L/M ratio baseline | 0.16 ± 0.02 [a] | 0.47 ± 0.07 [b] | 0.79 ± 0.06 [c] | *<0.0001* |
| L/M ratio Wk 10 | 1.41 ± 0.10 [a] | 1.50 ± 0.15 [a] | 0.88 ± 0.02 [b] | *0.001* |
| *p#* | *< 0.0001* | *0.0005* | *0.2671* | |

L,lactulose; M,mannitol; Wk, weeks of experiment. Values are expressed as means ± SEM (n=8). *Mean values within a row with unlike superscript letters were significantly different ($p<0.05$); Tukey's post hoc ANOVA statistical analysis. [#] The p-values refer to a paired t test between baseline and week 10.

*Lipid profile of SCFA and fecal pH*

The SCFA profile of acetate, propionate and butyrate was evaluated in the feces collected from the colon at the end of the experiment and the results are shown in **Figure 2**. The level of total SCFA, acetic acid, butyric acid and acetate/propionate ratio were higher in the Kefir group (p=0.019, **Figure 2A**; p=0.014, **Figure 2B;** p=0.037, **Figure 2D;** and p=0.0002**, Figure 2E** respectively) compared to PC group. The levels of propionate and fecal pH did not differ between the treatments (p=0.426, **Figure 2C** and p=0.337, **Figure 2F**, respectively).

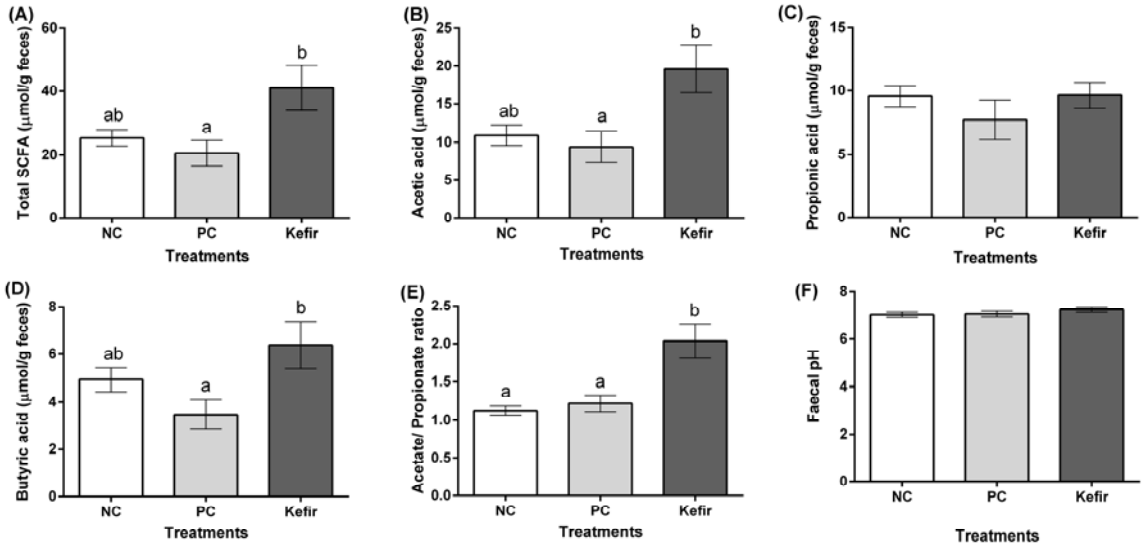


**Figure 2** – Concentration of SCFAs and pH in faeces of SHR rats with induced metabolic syndrome and supplemented with kefir. (A) Total SCFA; (B) Acetic acid; (C) Propionic acid; (D) Butyric acid; ((E) Acetate/propionate ratio; (F) Faecal pH. Values are means ± SD (n=8). [a,b,c] Differences among treatments were indicated by different letters and were considered statistically significant at p<0.05; for statistical analysis a Tukey's post hoc ANOVA test was used.

*Histological analyses*

The results of the histological analyses of ileum tissue are presented in **Figure 3**. The use of kefir was able to increase the villous weight (p<0.0001, **Figure 3A**) and crypt depth (p<0.0001, **Figure 3B**) when compared to PC group, indicating an increase in the development of the mucosa. The crypt depth was not influenced by the consumption of kefir (p<0.0001, **Figure 3C**).

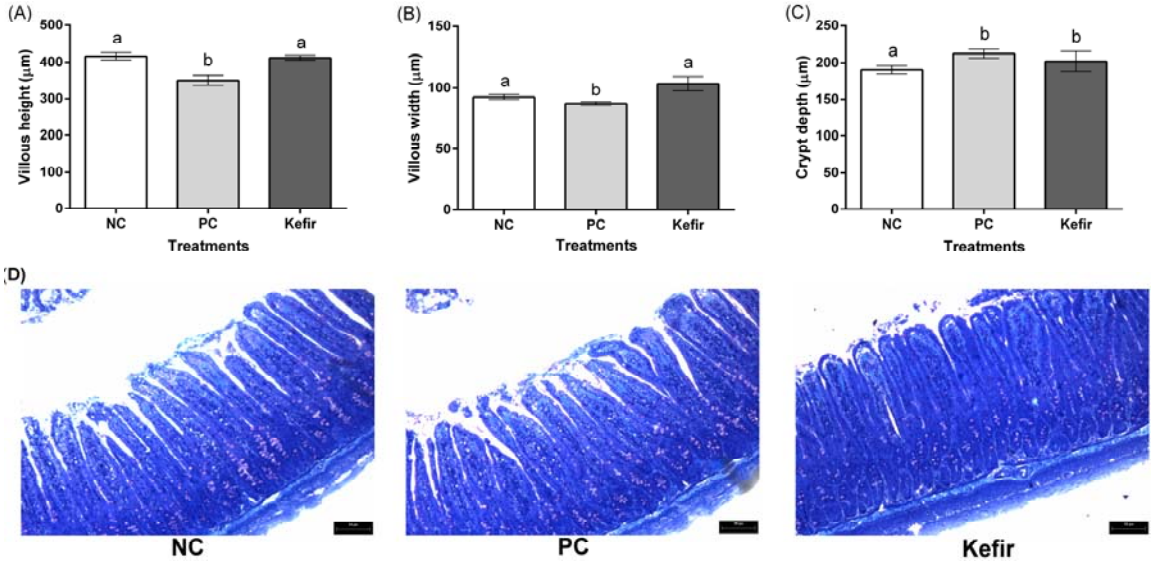


**Figure 3** – Photomicrographs of histological sections and morphometric analysis of small intestine of the study animals. Ileum segments were collected and processed for optical microscopy analysis at the end of the experiment. (A) Villous height; (B) Villous width; (C) Crypt depth; (D) The histological sections are represented in order: NC, negative control; PC, positive control; Kefir group; Bar =50µm. [a,b,c] Differences among treatments are indicated by different letters and were considered statistically significant at $p<0.05$; for statistical analysis a Tukey's post hoc ANOVA test was used. Values are means ± SEM (n=6).

Morphometric analysis performed in colon tissue showed that kefir consumption was unable to influence crypt depth ($p= 0.1068$, **Figure 4D**). The Kefir group show decrease in colonic mucosal thickness ($p<0.0001$, **Figure 4E**), when compared to PC group.

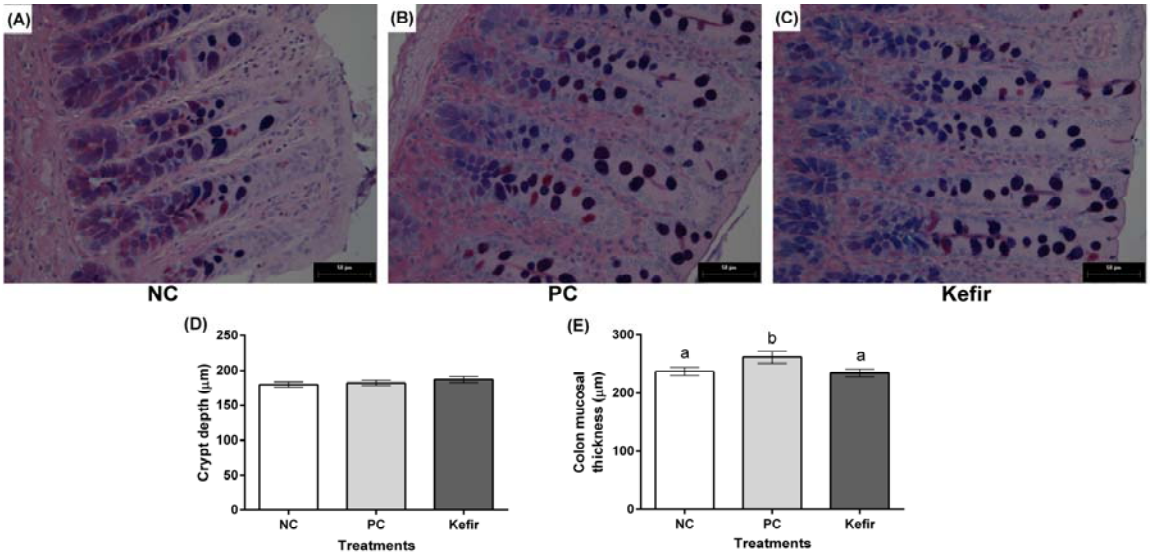


**Figure 4** –Photomicrographs of histological sections and morphometric analysis of large intestine of the study animals. Proximal colon segments were collected and processed for optical microscopy analysis at the end of the experiment. The sections were stained with PAS/Alcian Blue. (A) The histological sections are represented in order: NC, negative control; PC, positive control; Kefir group; Bar =50µm. (B) Crypt depth; (B) colon mucosal thickeness; [a,b,c] Differences among treatments are indicated by different letters and were considered statistically significant at $p<0.05$; for statistical analysis a Tukey's post hoc ANOVA test was used. Values are means ± SEM (n=6).

## 7.5. Discussion

A rich microbial complexity and putative benefits derived from consumption, make kefir a suitable source of potential probiotic microorganisms. However, there are few studies that relate the consumption of kefir with the maintenance of MetS. Our study is the first that assessed intestinal permeability, plasma lipopolysaccharide, short-chain fatty acids in feces, histological and morphometric analysis in the intestinal mucosa in an animal model of MetS both with and without supplementation with kefir.

Animals induced to MSG with MetS showed a significant reduction in the final body weight and lower weight gain at the end of the study when compared to NC group. According to Leguisamo *et al*. (2012), MSG-treated animals, compared to controls, may have a lower absolute weight, also we found in the MetS, which has been proposed to be a

result of decreasing growth hormone (GH) secretion, and lean mass may be decreased MSG in animals, and consumption of kefir did not influence this process.

The consumption of kefir did not affect plasma total cholesterol and HDL-c levels. However, the use of kefir significantly reduced plasma triacylglycerol levels compared to NC and PC groups. According to Leguisamo *et al*. (2012) the use of MSG in genetically hypertensive rats led these animals to progressively increase body adiposity and hypertriglyceridemia; besides, developing and maintaining insulin resistance, low HDL-cholesterol, high blood pressure levels, and inflammation, being considered a model of MetS. Supplementation with kefir was able to reduce hypertriglyceridemia, although it was not influenced in plasma cholesterol. Huang and coworkers (2013) shown a significant reduction in cholesterol and triglyceride levels in plasma and liver were observed in hypercholesterolemic rats treated with *Lactobacillus plantarum* Lp27 was isolated from Tibetan kefir grains and was fed to hypercholesterolemic rats. In another study, *Lactobacillus plantarum* MA2 isolate from Chinese traditional Tibet kefir were fed rats withcholesterol-enriched experimental diet. *L. plantarum* MA2 strain have hypocholesterolemic effect and also increasing the probiotic count in the intestine, demonstrating the probiotic effect on lipid metabolism and intestinal health (Wang et al., 2009).

We report that the consumption of kefir was able to reduce plasma LPS levels in rats. LPS is a natural constituent of Gram-negative bacteria and therefore it is a strong inducer of inflammatory response. LPS is involved in the release of several pro-inflammatory cytokines that are key factors triggering insulin resistance and thus it has been linked with the early development of metabolic diseases (Cani et al., 2007). Lower levels of LPS may have contributed to lower insulin resistance observed in our animals supplemented with kefir (data not shown).

There is a substantial evidence to suggest that certain probiotics can modulate systemic and mucosal immune function, stimulating mucus and antimicrobial peptides production, enhancing tight junction protein expression and/or localization, alter gut microbiota, and exert metabolic effects on the host in a strain- and dose-dependent manner (Bengmark, 2013). Animals supplemented with kefir had reduced intestinal permeability. The intestinal permeability is usually based on the ratio of two excretion probes: lactulose and mannitol, both of which are markers of small intestinal permeability. An increase in the L/M ratio is commonly observed in the presence of organic diseases. It can result from

increased lactulose excretion and/or reduced mannitol excretion, which may reflect inflammation of the intestinal mucosa, leading to abnormal villous morphology e.g. atrophy (Teixeira et al., 2012). The NC and PC groups showed increased intestinal permeability (increased L / M ratio), and kefir group did not show this increase in 10-week study, clearly showing the protective effect of probiotic kefir in intestinal mucosal. The morphometric analysis of the small intestine confirms these findings, showed that the consumption of kefir was related to greater villous height and villous with compared to NC and PC groups.

Chen and co-workers (2012) investigated the effect of *Lactobacillus kefiranofaciens* M1 isolated from kefir grains on germ-free mice with colitis. The results showed that continuous oral administration of this strain for 2 weeks was able to increase the ileum villous length and crypt depth of the germ-free mice. Therefore, the effect reinforcing or restoring epithelial barrier functionality might be a possible mechanism whereby *Lactobacillus kefiranofaciens* M1 is able to reduce colitis-like diseases. According to the results obtained in our study, better integrity of the intestinal mucosa of animals supplemented with kefir explains lower plasma levels of LPS and intestinal permeability. Higher endotoxin uptake could occur due to reduced expression of proteins of the epithelial tight junctions, such as zonulin and occludin, contributing to the deregulation of paracellular transport and increased intestinal permeability (Cani, Delzenne, Amar, & Burcelin, 2008). Additionally, the diet supplemented with kefir has a beneficial influence on intestinal microbiota. It has also been suggested that changes in the gut microbiota can influence the intestinal permeability and LPS levels (Teixeira et al., 2012).

Probiotics such as kefir that target the colon affect its environment enhancing SCFA production and fecal pH. The production of SCFA is determined by a number of factors, including the numbers and types of microbiota present in the colon, substrate source, and gut transit time. Absorption of SCFA in the cecum and the colon is a very efficient process with only 5% to 10% being excreted in the feces (Roberfroid, 2007). In agreement with the literature, the fecal SCFA production supplemented with kefir is in the order of acetate > propionate > butyrate (Wong, de Souza, Kendall, Emam, & Jenkins, 2006). In addition, we found that the consumption of kefir was able to increase the concentration of fecal butyrate, acetate and total SCFA. *In vitro* and *in vivo* studies have shown that butyrate is the preferred energy substrate and stimulates cell proliferation and differentiation in normal colonocytes (Roberfroid, 2007). Higher level of fecal butyrate observed in animals supplemented with kefir may have contributed to better mucosal integrity and lower levels

of LPS found in the kefir group. In our study, animals that consumed kefir showed intestinal mucosa that was more preserved, with higher villi in small intestine. However, no differences were found in the large intestine.

The short-chain fatty acids, how acetate and propionate have been proposed to have opposing effects in hyperlipidemia. Once absorbed, 50% to 70% of acetate is also taken up by the liver and is the primary substrate for cholesterol synthesis. On the other hand, propionate is also a substrate for hepatic gluconeogenesis and it has been reported that this acid inhibits cholesterol synthesis in hepatic tissue. In our study, we found a high ratio acetate / propionate group in kefir, may justify the absence of hypocholesterolemic action of kefir in these animals.

The ability of probiotics to reduce blood pressure has been found to be through the fermentation of food products in order to release bioactive peptides, such as the angiotensin-converting enzyme (ACE) inhibitory peptides that play a crucial role in the renin-angiotensin system (Lye, Kuan, Ewe, Fung, & Liong, 2009). Unlike previous studies, the consumption of kefir was unable to reduce the blood pressure of the animals and high pressure was maintained during the entire experimental period.

Taken together, our findings demonstrate for the first time that kefir supplementation in SHR with MetS for 10 weeks results in reduciton of plasma triglylglycerol, LPS levels and intestinal permeability, increase total SCFA, especially butyric acid and improve intestinal mucosal health. Therefore, kefir represents a promising potential in the management of MetS, at the same time being a low-cost and easily accessible probiotic product. It is noteworthy to mention that the gut microbiota can directly or indirectly be related to the evaluated parameters and thus may have contributed to the results obtained in our study. Further studies are necessary to explore the effect of kefir administration on gut microbiota composition and the role of gut microbes in the intestinal immune system and metabolism modulation, before the clinical application of kefir in MetS patients.

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**Competing interests**

The authors declare no conflict of interest.

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## 8. ARTICLE 4: Evaluation of the subchronic toxicity of kefir by oral administration in Wistar rats

*Evaluación de la toxicidad subcrónica del kéfir por administración oral en ratas Wistar*

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**Running title**: *Safety assessment of kefir*

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## 8.1. Abstract

*Introduction:* Kefir is obtained by fermentation of milk with complex microbial populations present in kefir grains. Several health-promoting benefits have been attributed to kefir consumption.

*Objective:* The objective of this work was to conduct a subchronic toxicity study, offering the rats normal or high-doses of kefir and evaluating growth, hematology and blood chemistry, as well as assessing bacterial translocation and the integrity of the intestinal mucosa of animals.

*Methods:* Wistar rats were randomly divided into three groups (n=6/group): control group received 0.7 mL of water, kefir group received 0.7 mL/day of kefir, (normodose), and Hkefir group received 3.5 mL/day of kefir (fivefold higher dose). Feeding was carried out by gavage. The animals were housed in individual cages and maintained under standard conditions for 4 weeks.

*Results:* The normodose and high-dose of kefir supplementation did not harm the animals since growth, hematology and blood chemistry in rats, as well as the potential pathogenicity in tissues were within normal limits, demonstrating that consumption of normodose and high-dose of kefir are safe. In addition, administration of the normodose of kefir reduced cholesterol levels and improved the intestinal mucosa of the rats.

*Conclusion:* These results demonstrate that the consumption of kefir is safe. Importantly, while damages are not seen for the high-dose, the normodose consumption is recommended due to the pronounced beneficial effects, as safety is concerned.

**Keywords***: Kefir, toxicity, safety, histological analysis, bacterial translocation*

## 8.2. Resumen

*Introducción:* El kéfir es obtenido por fermentación de la leche con una población microbiana compleja presente en sus granos. Al consumo de kéfir se le atribuyen múltiples efectos beneficiosos sobre la salud.

*Objetivo:* Evaluar la toxicidad subcrónica del kéfir en ratas Wistar, administrado por vía oral en dosis normal (normodosis) y sobredosis. Se evaluaron además, los parámetros de peso corporal, hematología, química sanguínea, translocación bacteriana e integridad de la mucosa intestinal.

*Métodos:* Se conformaron tres grupos de seis animales de manera aleatoria: grupo control, recibió 0.7 mL de agua; grupo kéfir recibió 0.7 mL/día de kéfir (normodosis) y grupo Hkéfir recibió 3.5 mL/día de kéfir (dosis cinco veces superior). La administración se llevó a cabo mediante sonda. Los animales se alojaron individualmente, y se mantuvieron bajo las mismas condiciones de manejo y alimentación durante 4 semanas.

*Resultados:* La administración de kéfir en dosis normal y sobredosis no afectó los parámetros evaluados en los animales, el peso corporal, indicadores hematológicos, de química sanguínea, y la patogenicidad potencial en los tejidos se encontraron dentro de límites normales, lo que demostró que el consumo de kéfir en dosis normal y sobredosis es seguro. Además, se evidenció que la administración de normodosis de kéfir redujo los niveles de colesterol y mejoró la mucosa intestinal de las ratas.

*Conclusión:* Se demostró que el consumo de kéfir es seguro. Destacar que, la administración de sobredosis no evidenció daños, no obstante, se recomienda el consumo de normodosis, debido a los marcados efectos beneficiosos y de seguridad.

**Palabras clave***:* kéfir, toxicidad, seguridad, análisis histológico, translocación bacteriana

## 8.3. Introduction

Fermented dairy products have been consumed by humans for thousands of years. Kefir is a drink obtained by fermenting milk with kefir grains, which contain bacteria and fungi that coexist in a complex symbiotic association [1]. When inoculated into a milk matrix, kefir grains produce an acidified fermented beverage that is self-carbonated, which contains mainly lactic acid and small amounts of alcohol and exopolysaccharides. Furthermore, bioactive peptides [3], antibiotic components [4], and numerous bacteriocins are produced [5].

Microorganisms including probiotics present in fermented milks, are associated with human helath benefits. This functional food is considered a probiotic because it contains live microorganisms that confer health benefits when administered in appropriate amounts [6]. The microbial strains present in kefir beverage often belong to species from the genera *Lactobacillus, Bifidobacterium* and *Saccharomyces*, acetic acid bacteria, and several genera of yeasts, whose health benefits have been well characterized [7].

Kefir consumption has been associated with several health-promoting properties, such as antimicrobial [8], anti-inflammatory [9], reduction of cholesterol and triglycerides plasma levels [10] and has also been shown to exert beneficial effect on gut health [11]. Kefir for centuries has been empirically used in many eastern European regions to treat different gastrointestinal diseases. Kefir has gained interest in the scientific community due to its health benefits against numerous diseases and infections [12].

Among probiotic functional foods, kefir stands out because of its low cost; it can be produced at home and can easily be incorporated into the diet. However, little attention has been payed to the safety concern with the use of kefir. The information on the safe levels of kefir intake or the amount that needs to be consumed and the time required to exert beneficial health effects are sparse in the literature. Based on the widespread worldwide kefir consumption, which is increasing daily due to the globalization of food habits, such safety studies are urgently needed.

In general, probiotics have been considered as safe. There are however some theoretical adverse risks regarding the use of beneficial microbes in humans. They include the potential for translocation and negative impact on gastrointestinal physiology and function, including metabolic and physiologic effects. Finally, there is

also the potential for antibiotic resistance transfer within the gastrointestinal tract from commensal or probiotic bacteria to other bacteria or pathogens [6, 13].

Therefore, the objective of our study was to conduct a subchronic toxicity assay with kefir using rat-animal-model. We offered the animals different doses of kefir for 4 weeks and thereafter evaluated their growth, hematology, and blood chemistry. The potential infectivity and pathogenicity (translocation and mucosal histology) of kefir were also assessed.

## 8.4. Materials and Methods

### Kefir preparation

Kefir particles (grains of kefir, obtained from a private household in Viçosa, Minas Gerais, Brazil) were washed with distilled water and inoculated in whole cow's milk during incubation at room temperature. The kefir beverage was prepared by inoculation of 5% (wt/wt) kefir grains into pasteurized milk. After incubation at 25–28 °C for 24 hours, the grains were separated from the fermented milk by filtration through a plastic sieve, washed, and kept for next preparation [11]. This process was repeated daily throughout the 4 weeks of the experimental period. Animals received fresh milk and kefir drink every day.

The fresh kefir offered to the animals presented the following physicochemical composition: pH 4.10 ± 0.10; high acidity, 0.461 ± 0.06 g/100 g of lactic acid; lipids 3.30 ± 0.16 g/100 g, proteins 3.00 ± 0.01 g/100 g. The lactic acid bacteria (LAB) were present in kefir at the levels of $2.78 \times 10^7$ CFU/mL and yeasts at $2.94 \times 10^8$ cell/mL, as determined by selective agar plating.

### Animals

Eight-week-old male and female Wistar rats, which were supplied by the Experimental Animal Center, National Center for Animal and Plant Health, Mayabeque, Cuba, were used in this study. They received commercial standard chow (16.0% protein, 56.0% carbohydrate, 2.0% fat, 5.3% cellulose, and 5.0% vitamins and minerals) and tap water *ad libitum* for 1 week to allow adaptation of the animals. They were housed three to four rats per polycarbonate cage with softwood chips as bedding, in a barrier-sustained animal room, air-conditioned at 23–25 °C and 50–60% humidity, on a 12 h light/dark cycle.

**Experimental Design**

Eighteen animals were randomly divided into three groups. Each group consisted of three male (body weight: 210.0 ± 0.35 g) and three female (body weight: 180 ± 0.50 g) rats. The animals were placed in individual cages under controlled conditions. The experimental design is included (**Figure 1**):

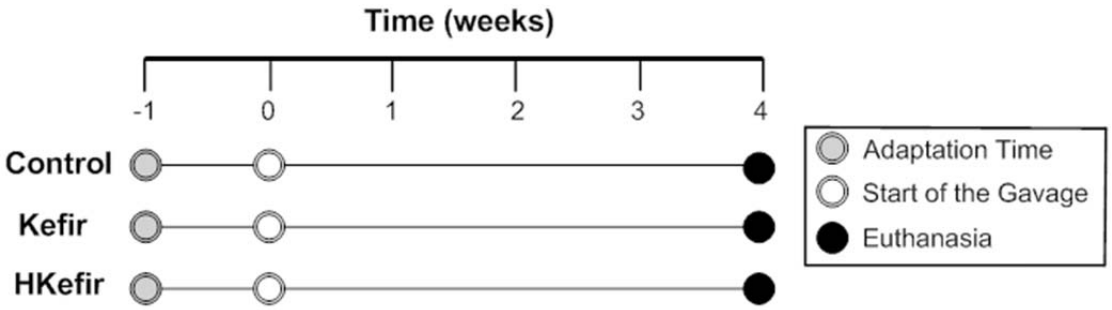


**Figure 1** – Experimental design of the study.

- Control group (Control): standard diet plus oral administration of distilled water at a dose of 0.7 ml/animal/day by gavage;
- Normal dose of kefir group (kefir): standard diet plus oral administration of kefir at a dose of 0.7 ml/animal/day ($9.8 \times 10^6$ CFU/mL or $4.29 \times 10^7$ CFU/ kg body weight/day) by gavage;
-High dose of kefir group (Hkefir): standard diet plus oral administration of kefir at the dose of 3.5 ml/animal/day ($4.9 \times 10^7$ CFU/mL or $2.1 \times 10^8$ CFU/ kg body weight/day) by gavage. This group received a fivefold higher dose of kefir.

Body weight was measured weekly. At the end of week 4, all of the animals were anesthetized with ethyl ether according to the Guidelines for Ethical Conduct in the Care and Use of Nonhuman Animals in Research Ethical [14]. At the end of the experiment, the organs (spleen, liver, and mesenteric lymph nodes) were collected and weighed under aseptic conditions and inserted into a sterile falcon tube for further analysis of bacterial translocation. Blood was collected by cardiac puncture for hematologic and biochemical analysis. For histological evaluation, samples of the liver, small intestine, cecum and colon were collected and fixed in 10% buffered formalin.

**General health status**

Throughout the experimental period, changes in behavior and activity, treatment-related illness or death, and differences in hair luster between the treatments and control groups were monitored.

**Internal organ indices**

Liver, heart, kidney and spleen were collected and weighted immediately after euthanasia. The organ index values were derived from the ratio between weights of the internal organs (mg) of each animal over its final body weight (g).

**Hematology and blood biochemistry**

The blood was collected by cardiac puncture from anesthetized animals. Blood samples were centrifuged at 700 x g for 10 minutes to obtain serum and thereafter were frozen at -20 °C. Total cholesterol and triacylglycerol were determined using commercial diagnostic test kits (Bioclin®, Diagnostics®, Belo Horizonte, Brazil). The blood samples were collected and transferred to lead-free polyethylene tubes containing EDTA. A cell counter was applied for hematocrit, by the microhematocrit method using heparinized capillary tubes; total leukocyte counts using a Neubauer chamber and Giemsa staining blood smears for differential cell counting by optical microscopy.

**Bacterial translocation**

Liver, spleen and mesenteric lymph nodes were collected and weighted under strict aseptic conditions to avoid any cross-contamination. The tissues were separately plated in three media. Blood agar based medium (Oxoid, Unipath Ltd., Basingstoke, Hampshire, UK) was prepared according to the manufacturer's instructions. The plates were incubated at 37 °C aerobically for 24 h to evaluate the presence of bacteria and yeasts. Sabouraud Maltose Agar (BioCen, Havana, Cuba), plates were incubated at 30 °C aerobically for 48 h to assess the presence of yeasts. The MRS agar (Himedia®, Mumbai, India) plates were incubated at 37 °C anaerobically for 48 h to control the presence of bacterial colonies.

**Histological analysis**

Immediately after euthanasia, liver, ileum, caecum and proximal colon samples were excised and rinsed with ice-cold physiological saline for histological evaluation. Tissue samples of approximately 0.5 cm in length were excised from the ileum (2 cm proximal to the caecum), caecum (middle portion), and colon (2 cm distal to the caecum), and fixed in 10% buffered formalin [15]. After dehydration in an increasing gradient of ethanol, tissue was embedded in paraffin and stained with routine histological hematoxylin and eosin for the histological analysis. Ten images per animal (60 images for each group) were captured and morphometric digital analysis for determining villous height, villous width and crypt depth in ileum tissue were performed according to the procedure described by Rosa et al.(2010) [16]. Crypt depth, mucosa thickness of the caecum and proximal colon samples were measured as described by Kabeir et al.(2008) [17]. The measurements were taken using the Image Pro-Plus® software system, version 4.5 (Media Cybernetics). In liver, hepatic parenchyma was classified as one of the following: hepatocyte cytoplasm or nucleus, hepatic sinusoids, degenerative hepatocytes, central vein, portal space, fatty deposition, and inflammatory infiltrate, according to Predes et al.(2009) [18] .

**Ethical aspects**

This project was approved by the Commission of Ethics in Animal Experimentation of the National Center for Animal and Plant Health, Mayabeque, Cuba.

**Statistical Analysis**

Results are presented as mean values with their standard deviation (SD). Statistical significance of the difference between groups was assessed by one-way ANOVA followed by Tukey's post hoc multiple comparison test and chi-square analysis using GraphPad Prism (GraphPad Software Inc., San Diego, CA); for statistical analysis $p < 0.05$ was considered statistically significant.

## 8.5. Results

### General health status and growth of the animals

During the experimental period, there was no noticeable change in activity, behavior or hair luster in any of the experimental groups. No diarrhea or other treatment-related sickness or death was recorded. At the end of the experimental period, all animals were alive and healthy. The consumption of the different doses of kefir by the animals did not affect weight gain of the animals during the experimental period (Figure 2).

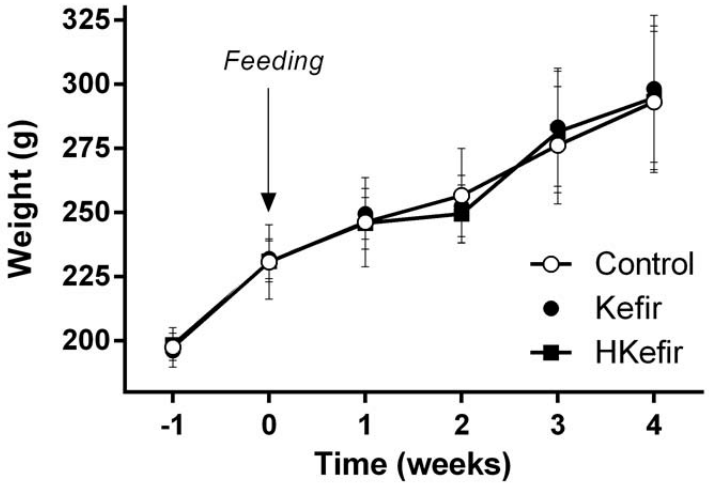


**Figure 2** – Effect of kefir consumption on the weight change during the experimental period. There were no significant differences in weekly weight gain among control group and the groups fed with normal dose and high dose kefir (p> 0.05; Tukey's post hoc ANOVA statistical analysis).

Table 1 shows the internal organ indices of the different animals groups.

**Table 1**. Effect on internal organ (liver, heart, kidney and spleen) indices of Wistar rats fed with normal dose and high dose of kefir.

| Organs | **Control** | **Kefir** | **HKefir** | *p** |
|---|---|---|---|---|
| *Liver* | 31.66 ± 7.36 | 29.84 ± 3.02 | 29.70 ± 3.02 | *0.560* |
| *Heart* | 3.44 ± 0.55 | 3.62 ± 0.44 | 3.62 ± 0.44 | *0.635* |
| *Kidney* | 7.32 ± 0.93 | 7.32 ± 0.68 | 7.32 ± 0.68 | *0.957* |
| *Spleen* | 2.43 ± 0.29 | 2.34 ± 0.29 | 2.31 ± 0.27 | *0.628* |

*There were no significant differences in the organ indices (means ± SD, n=6) between the control group and the groups fed with normal dose and high dose of kefir (p>0.05; Tukey's post hoc ANOVA statistical analysis).

The indices of the liver, heart, kidney and spleen revealed no significant differences in the ratio of organ weight/live weight between the groups fed normal dose or high dose of kefir or the control group at the different time points ($p>0.05$).

**Hematology / blood biochemistry**

The effects of supplementation with different doses of kefir for 4 weeks on hematological and biochemical parameters were investigated in this study (Table 2).

**Table 2**. Hematology and blood biochemistry measurements of rats orally administrated with normal dose and high dose of kefir for 4 weeks.

| Parameter | **Control** | **Kefir** | **Hkefir** | *p* |
|---|---|---|---|---|
| Total cholesterol (mmol/L) | $1.82 \pm 0.07^{a}$ | $1.47 \pm 0.14^{b}$ | $2.01 \pm 0.20^{a}$ | *0.017* |
| Triacylglycerol (mmol/L) | $0.86 \pm 0.09$ | $0.93 \pm 0.12$ | $0.74 \pm 0.06$ | *0.437* |
| Hematocrit (L/L) | $37.00 \pm 1.58$ | $39.00 \pm 4.18$ | $36.50 \pm 1.09$ | *0.602* |
| Total leukocytes (x $10^9$/L) | $10.18 \pm 2.54$ | $8.51 \pm 2.44$ | $8.23 \pm 2.05$ | *0.494* |
| Neutrophils (x $10^9$/L) | $1.75 \pm 0.44$ | $1.87 \pm 0.65$ | $1.37 \pm 0.35$ | *0.639* |
| Lymphocytes (x $10^9$/L) | $8.30 \pm 2.07$ | $6.47 \pm 1.95$ | $6.69 \pm 1.32$ | *0.368* |
| Monocytes (x $10^9$/L) | $0.07 \pm 0.03$ | $0.02 \pm 0.01$ | $0.04 \pm 0.02$ | *0.524* |
| Eosinophils (x $10^9$/L) | $0.14 \pm 0.07$ | $0.13 \pm 0.03$ | $0.13 \pm 0.05$ | *0.971* |

Values are expressed as means ± SD, n=6. $^{a,b}$ Mean values within a row with unlike superscript letters were significantly different ($p<0.05$; Tukey's post hoc ANOVA statistical analysis).

After 4 weeks of treatment, kefir group showed a reduction in total cholesterol plasma levels when compared to control and HKefir groups ($p=0.017$). In this period, the levels of triacylglycerol, hematocrit, total leukocytes, and leukocytes fractions remained unchanged ($p>0.05$).

**Bacterial translocation**

The incidence of bacterial translocation in rats which received orally different doses of kefir is assessment in liver, spleen and mesenteric lymph nodes. For this analysis, positive animal translocation was defined as an animal that had at least one tissue sample containing one or more viable bacterial cells. None of the animals were positive for the translocation ($p>0.05$, data not shown).

**Histological measurements**

Macroscopic evaluation revealed that the animals did not show significant alterations in the liver, spleen, heart, kidney, ileum, cecum, and colon. The morphological analysis did not reveal any histopathological alterations in the liver, as well as in lipid deposition among animals from different treatment groups (data not shown).

Histological evaluations were performed with the three parts of the intestine: ileum, cecum and colon. Four weeks of kefir administration resulted in modulation of the distal small intestine (Figure 3).

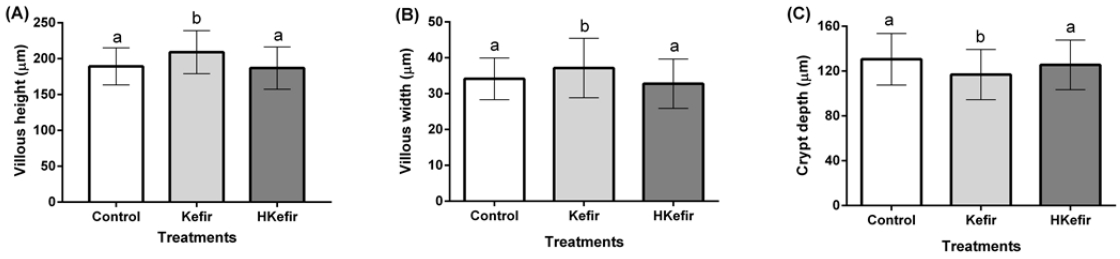


**Figure 3 –** Morphometric analysis of small intestine of the animal groups studied. Ileum segments were collected and processed for optical microscopy analysis at the end of the experiment. (A) Villous height; (B) Villous width; (C) Crypt depth; Values are the means ± SD (n = 6). [a,b,c] Differences among treatments were indicated by different letters and were considered statistically significant when $p<0.05$; Tukey's post hoc ANOVA statistical analysis.

During this period, the kefir group showed greater villous height and villous width when compared to the control and Hkefir groups ($p<0.0001$; Figure 3A and 3B). On the other hand, kefir group showed lower crypt depth ($p<0.0001$; Figure 3C).

The influence of kefir supplementation on the cecum and colon of animals are shown in Figure 4.

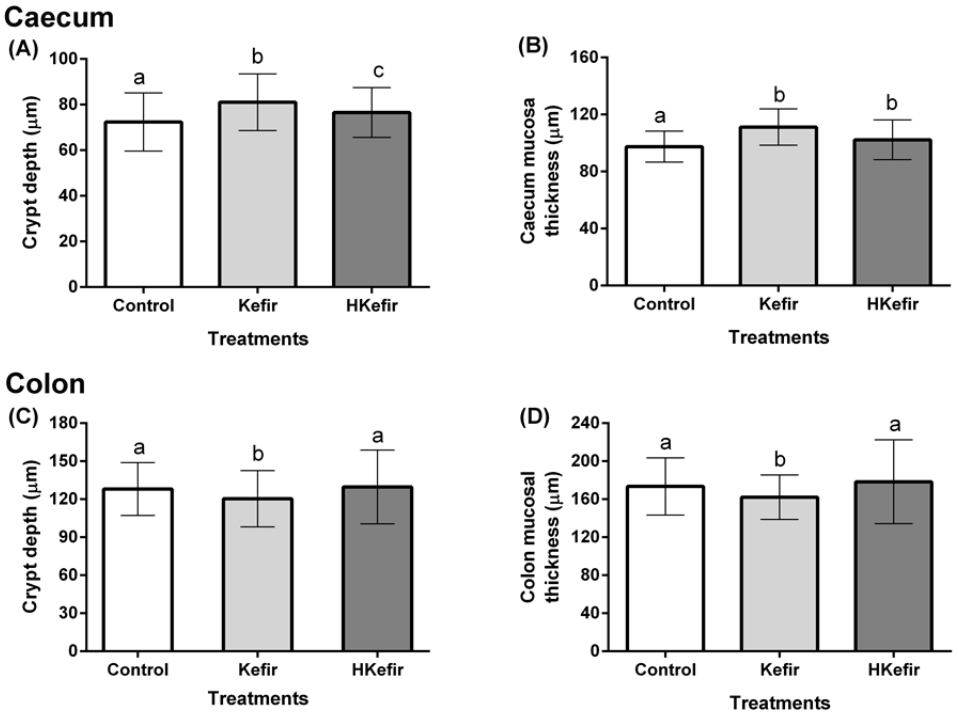


**Figure 4 –** Morphometric analysis of large intestine of the animal groups studied. Caecum and proximal colon segments were collected and processed for optical microscopy analysis at the end of the experiment. (A) Crypt depth in the caecum; (B) caecum mucosal thickness; (C) crypt depth in proximal colon; (D) colon mucosal thickness. Values are means ± SD (n=6). [a,b,c] Differences among treatments were indicated by different letters and were considered statistically significant when $p<0.05$; Tukey's post hoc ANOVA statistical analysis.

After 4 weeks, kefir group had the highest crypt depth ($p<0.0001$; Figure 4A). The animals treated with kefir (kefir and Hkefir groups) had higher mucosal thickness in cecum when compared to the control group ($p<0.0001$; Figure 4B). In the initial portion of the colon, the animals that consumed normal dose of kefir showed lower crypt depth ($p<0.0001$; Figure 4C) and colon mucosal thickness ($p=0.0018$; Figure 4D).

### 8.6. Discussion

Fermented products including functional foods, are consumed by humans widely. They often represent an important dietary component in different geographical regions. Kefir is considered a functional food with probiotic properties that provides health benefits to the host. Indeed, the consumption of kefir has been increasing worldwide. However, there is a dearth of information on the safety of kefir consumption, especially the amount and length of consumption. Our study is the first to assess the *in vivo* safety of kefir supplementation in animal model. We conducted a

study to evaluate the effect of two different doses of kefir in a subchronic toxicity assay. General health, organ weight index, hematology and blood biochemistry, and traditional histology were assessed.

After 4 weeks of consumption of different doses of probiotic kefir, the animals did not show differences in bodyweight or internal organ indices. The administration of the normal dose or high dose of kefir did not adversely affect the general health of the animals. In probiotic toxicity studies, behavioral, as well as activity, increasing organs size, especially splenomegaly and hepatomegaly, are the first indicators of undesirable effects. Particularly, the ratio of the spleen weight to body weight is considered to be an indicator of spleen inflammation by enteropathogenic bacteria [19]. However, in our study, we did not detect any toxicity signs related to kefir consumption

The consumption of fermented dairy products including kefir has been proposed as a strategy to reduce levels of circulating cholesterol and to improve lipid profile in humans and animals [20]. In this study, the consumption of kefir at the normal dose was able to reduce the total cholesterol levels. According to Hosono et al. [21], a high count of LAB in kefir ensures binding of cholesterol by up to 33%, probably due to the direct action of microbiota through their metabolic products on total cholesterol. Such beneficial effects of kefir on cholesterol metabolism may be due to the production of short chain fatty acids [22] and by the deconjugation of bile acids [12] by microorganisms. Similarly, significant reductions in cholesterol level in the plasma and liver were observed in hypercholesterolemic rats treated with *Lactobacillus plantarum strains* Lp09 and Lp45 [10] and *Lactobacillus acidophilus* LA15, *Lactobacillus plantarum* B23, and *Lactobacillus kefiri* D17 [23] isolated from kefir grains. Thus, there is strong evidence supporting the functionality of kefir in the control of cholesterol level.

According to Kabeir and coworkers[17], infectivity and pathogenicity are two important components in safety studies on probiotic bacteria and are expressed as the degree of bacterial translocation. In our study, supplementation of rodents with normal or high doses of kefir did not cause bacterial translocation. Bacterial translocation is defined as the passage of viable bacteria from the gastrointestinal tract through the mucosal epithelium to other tissues. It can occur in cases of physical disruption of the mucosal barrier, thus initiating the first step of infectivity, and in the pathogenesis process of many opportunistic indigenous microbes. If physical disruption of the mucosal barrier occurs, the liver is the first organ to be compromised because of its

direct connection through the portal blood. The morphological analyses did not reveal any histopathological alterations in the liver tissue of the study animals.

Some beneficial mechanisms of kefir include competition with pathogenic bacteria for the adhesion sites and strengthening of the physical and immunological barrier function of the intestine. In the present study we performed the histological analyses on the ileum, caecum, and colon. We found that the mucosa, villous and the intestinal crypts were well defined and healthy since the supplementation with different doses of kefir did not result in damage to the intestinal mucosa. Histological evaluation was carried out to corroborate the activity of kefir on the preservation of the structure of the intestinal mucosa in the ileum. Here, the consumption of 0.7 mL/day kefir increased villous weight and width. Our results indicate that a strong hyperplasia process occurred in these groups. This would guarantee the cell turnover rate in order to compensate for the cell loss in the apical region of the villous. In the literature some studies show that in the small intestine, enterocytes generated from stem cells in the crypt base differentiate into absorptive cells and are finally lost from the tips of the villus, resulting in the replacement of lining cells every 2–3 days [16]. On the other hand, the high dose of kefir in our study did not conferred damage to the intestinal mucosa.

Similarly, the normal dose of kefir resulted in better results in the caecum since it increased crypt depth after 4 weeks of consumption. On the other hand, in kefir group we observed a decreased mucosal colon crypt depth and thickness when compared with the other treatments. Our histological analyses of the intestinal mucosa of the animals corroborate the results of the bacterial translocation. The probiotics could prevent the attachment of pathogens and stimulate their removal from the infected intestinal tract. The mechanisms of these beneficial effects are related to the exclusion of pathogenic bacteria by direct antagonism, competition for nutrients, adhesion receptors, and stimulation of host immunity [24].

In the present study, the animals received 0.7 mL of kefir as the normal dose and a dose 5 times higher (3.5 mL) which was considered a high dose. By extrapolating to the human diet and considering an adult man of approximately 70 kg bodyweight, 0.7 mL/day/animal of this probiotic represents a daily dose of 200 mL of kefir/human/day, a quantity that is easily incorporated into a diet. Likewise, the high-dose kefir for human consumption represents a daily consumption of 1 000 mL of kefir, which is unlikely to be incorporated into the human diet.

Taken together, we conclude that kefir supplementation with normal dose and high dose for 4 weeks in Wistar rats did not demonstrate harmful effects on the animals, as determined by growth, hematology, and blood chemistry in rats, as well as the potential pathogenicity in tissues. These findings clearly demonstrate that consumption of both the normal dose and high dose of kefir are safe. The results emphasize that, although no damages in the mucosa were seen at the high-dose-consumption of kefir, the normal dose is recommended due to the most pronounced beneficial effects, as safety is concerned.

**Acknowledgements**

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## 9 General Conclusions

This is the first study that evaluated the effect of supplementation with Kefir in animal model induced to MetS. The results of this study showed that daily consumption of probiotic kefir for 10 weeks was able to modulate important parameters for determining the occurrence of MetS, such as central adiposity, insulin resistance and plasma triglyceride levels. Additionally, the supply of kefir to mice with MetS was able to exert an important effect intestinal level. Kefir reduced intestinal permeability, plasma LPS, increase SCFA and improve gut health of the animals. In the present study, the animals received 1 mL of kefir by gavage. Making an extrapolation to the human diet and considering the adult man of approximately 70 kg body weight, the volume of this probiotic drink for body weight represents a daily dose of 230 mL of kefir, a quantity easily incorporated in a diet. Our study toxicity test on animals demonstrated that consumption of both the normal dose and high dose of kefir are safe. The results emphasize that, the normal dose is recommended due to the most pronounced beneficial physiological effects. Thus, kefir is a low cost probiotic, easily incorporated into the diet, which can positively assist in prevention and risk reduction of MetS. Controlled clinical trials with subjects with the MetS are needed and should be the next step to better understand the physiological effects of kefir on human feeding.

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## CERTIFICADO

A Comissão de Ética para Uso de Animais - CEUA/ UFV certifica que o processo n.° 107/2011, intitulado *"Avaliação do consumo de Kefir nos parâmetros metabólicos, hormonais e imunes em ratos SHR induzidos a síndrome metabólica"* coordenado pela professora Maria do Carmo Gouvêia Pelúzio, do Departamento de Nutrição e Saúde, está de acordo com o Código de Ética Profissional do Médico Veterinário, com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal/Sociedade Brasileira de Ciência em Animais de Laboratório (COBEA/SBCAL) e com a legislação vigente, tendo sido aprovado por esta Comissão em 25/05/2012.

## CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 107/2011, *"Evaluation of the use of the kefir in metabolic, hormonal and immunity parameters of SHR rats induced metabolic syndrome"*, is in agreement with the Medical Veterinary Professional Ethics Code, with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation/Brazilian of Animal Science Laboratory Society (COBEA/SBCAL) and with actual Brazilian legislation. This Institutional Commission on May 25, 2012 approved this process.

Viçosa, 25 de maio de 2012

***Professor Cláudio César Fonseca***
Comissão de Ética para o Uso de Animais da UFV - CEUA
Coordenador